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Current and future strategies for the treatment of malignant brain tumors

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Abstract

Glioblastoma (GB) is the most common subtype of primary brain tumor in adults. These tumors are highly invasive, very aggressive, and often infiltrate critical neurological areas within the brain. The mean survival time after diagnosis of GB has remained unchanged during the last few decades, in spite of advances in surgical techniques, radiotherapy, and also chemotherapy; patients' survival ranges from 9 to 12 months after initial diagnosis. In the same time frame, with our increasing understanding and knowledge of the physiopathology of several cancers, meaningful advances have been made in the treatment and control of several cancers, such as breast, prostate, and hematopoietic malignancies. Although a number of the genetic lesions present in GB have been elucidated and our understanding of the progressions of this cancer has increased dramatically over the last few years, it has not yet been possible to harness this information towards developing effective cures. In this review, we will focus on the classical ways in which GB is currently being treated, and will introduce a novel therapeutic modality, i.e., gene therapy, which we believe will be used in combination with classical treatment strategies to prolong the life-span of patients and to ultimately be able to control and/or cure these brain tumors. We will discuss the use of several vector systems that are needed to introduce the therapeutic genes within either the tumor mass, if these are not resectable, or the tumor bed, after successful tumor resection. We also discuss different therapeutic modalities that could be exploited using gene therapy, i.e., conditional cytotoxic approach, direct cytotoxicity, immunotherapy, inhibition of angiogenesis, and the use of pro-apoptotic genes. The advantages and disadvantages of each of the current vector systems available to transfer genes into the CNS are also discussed. With the advances in molecular techniques, both towards the elucidation of the physiopathology of GB and the development of novel, more efficient and less toxic vectors to deliver putative therapeutic genes into the CNS, it should be possible to develop new rationale and effective therapeutic approaches to treat this devastating cancer.

Keywords: Glioblastoma; Viral vectors; Suicide genes; Gene therapy; Clinical trials; Brain tumors

Contents

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1. Introduction

Tumors of the CNS have special features that make them different from other neoplasms in the body. Firstly, the distinction between benign versus malignant tumors is less evident in the CNS. Secondly, irrespective of histological classification, they can be highly malignant, depending on their anatomical location. Thirdly, they rarely metastasize outside of the CNS, although they often infiltrate into the surrounding brain parenchyma.

The incidence of intracranial neoplasms is $\sim$10–20 per 100,000, and they account for $\sim$2% of deaths in Western countries (Annegers et al., 1981; Counsell & Grant, 1998; Pobereskin & Chadduck, 2000). In adults, one-half of the brain tumors are primary, and the rest are metastatic (Annegers et al., 1981). Primary brain tumors have a bimodal distribution, with an early small peak in childhood at 5–9 years and the other, much higher, at 60–69 years (Sutherland et al., 1987). Although primary brain tumors are the second most common type of cancer in children under 15 years old (leukemia is the first) and the third leading cause of cancer-related deaths in adults under 34 years old, the majority of these tumors appear after the fifth decade of life. The white population has a higher incidence rate compared with other populations (McLendon et al., 1985). Asian populations (including some highly developed countries, such as Japan, Hong Kong, and Singapore) and African nations report lower rates. About 50% the primary neoplasms are gliomas and 50% of these gliomas are the most malignant variety, glioblastoma (Table 1). Meningiomas are the most common types of the non-gliomatous primary brain tumors, followed by schwannomas, which make up 20% and 10% of brain tumors, respectively. Certain histological tumor types characteristically occur mainly in specific age groups; examples include medulloblastoma in children and glioblastoma in the elderly. There is a slight predominance (55%) of primary brain tumors in males, with the exception of meningiomas, which are twice as common in females (Cole et al., 1989). The etiology of primary brain tumors is unknown and data on environmental risk factors is conflicting and unclear.

Pathological approaches have shown that at the time of the initial diagnosis, most malignant glioma tumors have spread more than 15 mm over the area that can be identified by magnetic resonance imaging (MRI) scan (Kelly et al., 1987; Greene et al., 1989). Several molecular mechanisms have been involved in the development of gliomas and their progression to more malignant tumors. For instance, the progression to high-grade glioma is associated with inactivation of the p53 tumor suppressor gene on chromosome 17p, as well as over expression of platelet-derived growth factor (PDGF) (Sidransky et al., 1992; Dunn et al., 2000). Furthermore, progression to glioblastoma involves amplification of the epidermal growth factor receptor (EGF-R) gene and the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Schlegel et al., 1994; Dunn et al., 2000).

Various studies have demonstrated that patients with glioblastoma are immunosuppressed (Roszman et al.,

Table 1
WHO histological classification of tumors of the CNS of neuroepithelial origin (Kleihues & Cavanee, 2000)

Class	Type	Subtype
Astrocytic tumors	1. Diffuse astrocytoma	1.1 Fibrillary astrocytoma
		1.2 Protoplasmic astrocytoma
		1.3 Gemistocytic astrocytoma
	2. Anaplastic astrocytoma	
	3. Glioblastoma	3.1 Giant cell glioblastoma
		3.2 Gliosarcoma
	4. Pilocytic astrocytoma	
	5. Pleomorphic xanthoastrocytoma	
	6. Subependymal giant cell astrocytoma	
Oligodendroglial tumors	1. Oligodendroglioma	
	2. Anaplastic oligodendroglioma	
Mixed gliomas	1. Oligoastrocytoma	
	2. Anaplastic oligoastrocytoma	
Ependymal tumors	1. Ependymoma	1.1 Cellular
		1.2 Papillary
		1.3 Clear cell
		1.4 Tanycytic
	2. Anaplastic ependymoma	
	3. Myxopapillary ependymoma	
	4. Subependymoma	
Choroid plexus tumors	1. Choroid plexus papilloma	
	2. Choroid plexus carcinoma	
Glial tumors of uncertain origin	1. Astroblastoma	
	2. Gliomatosis cerebri	
	3. Chordoid glioma of the third ventricle	
Neuronal and mixed neuronal-glial tumors	1. Gangliocytoma	
	2. Dysplastic gangliocytoma of the cerebellum (Lhermitte-Duclos)	
	3. Desmoplastic infantile astrocytoma/ganglioma	
	4. Dysembroyplastic neuroepithelial tumor	
	5. Ganglioglioma	
	6. Anaplastic ganglioglioma	
	7. Central neurocytoma	
	8. Cerebellar liponeurocytoma	
	9. Paraganglioma of the filum terminale	
Neuroblastic tumors	1. Olfactory neuroblastoma (esthesioneuroblastoma)	
	2. Olfactory neuroepithelioma	
	3. Neuroblastoma of the adrenal gland and sympathetic nervous system	
Pineal parenchymal tumors	1. Pinecytoma	
	2. Pineoblastoma	
	3. Pineal parenchymal tumor of intermediate differentiation	
Embryonal tumors	1. Medulloepithelioma	
	2. Ependymoblastoma	
	3. Medulloblastoma	3.1 Desmoplastic medulloblastoma
		3.2 Large cell medulloblastoma
		3.3 Medullomyoblastoma
		3.4 Melanocytic medulloblastoma
	4. Supratentorial primitive neuroectodermal tumor (PNET)	4.1 Neuroblastoma
		4.2 Ganglioneuroblastoma
	5. Atypical teratoid/rhabdoid tumor	

1991; Dix et al., 1999). Significantly, impaired delayed hypersensitivity responses to common antigens have been demonstrated in these patients. A number of immunosuppressive molecules, such as transforming growth factor-β (TGF-β), IL-10, and prostaglandin E_2, are reported to be involved in down-regulation of the cellular immune response in glioma patients. Extensive in vitro and in vivo studies support that increased production of TGF-β may result to tumor immune escape by induction of growth arrest and apoptosis in immune cells, by suppression of MHC II antigen expression, and by inhibition of the development cytotoxic T-lymphocytes (reviewed by Platten et al., 2001). The clinical presentation of brain tumors depends on the location of the tumor and its growth rate. Patients may present with symptoms either due to focal tissue destruction or because of edema and raised intracranial pressure. Glioblastoma is usually centered in the deep white matter of the cerebral hemispheres, most frequently in the frontal lobe. The frontal lobe tumors commonly manifest with mental and personality changes as an early symptom. Headache is the most common complaint and papilledema the most common physical finding in patients with hemispheric glioblastoma. The triad of headache, epilepsy and hemiparesis is seen in $<50\%$ of patients. The outcome for malignant glioma patients has not considerably improved during the last decades, despite technical advances in neurosurgery, radiotherapy, and chemotherapy. Survival is generally related to tumor histopathology, anatomic location, and age of patient. Age is usually a good prognostic factor, and younger glioma patients survive longer. The

average survival of glioblastoma patients with current therapeutic methods is <9 months.

2. Current treatment strategies for brain gliomas

Current treatments for malignant or high-grade gliomas rarely achieve long-term tumor control. Even when treated with aggressive combined surgery, chemotherapy, and radiotherapy, in patients with high-grade malignant brain glioma such as glioblastoma, recurrence occurs between 6 and 12 months and for anaplastic astrocytoma, within 18–36 months. In the following sections, we will review current treatment strategies for the treatment of glioblastoma (GB).

2.1. Surgery

Surgery plays a major role in the management of gliomas. Low-grade tumors should be resected to the limitation of clinical deficits. Both biopsy and resection are relevant concepts in malignant tumors, and clinical outcome is a higher goal in malignant brain tumors than is the extent of surgery. Tumor resection is also beneficial to alleviate mass effect. At diagnosis, tumors are usually localized and are <5 cm in diameter (Eck et al., 1996). It is thought that surgery for the treatment of GB can prolong the life of the patient for up to 6 months (Shand et al., 1999). GB tumors can be difficult to resect due to the lack of a defined tumor edge; the tumor may extend into normal-looking brain tissue, and/or can be localized near critical areas of the brain. Before surgery, the size and number of tumors must be considered, along with the general health of the patient. Tumors can be either completely or partially resected, or biopsied. It is thought that even partial resections are beneficial to the patients, as it improves body functions, relieves pressure in the brain, and disrupts the blood-brain barrier (Balmaceda, 2000). This allows for enhanced exposure to chemotherapeutic drugs. It also provides space for the tumor to grow, and pushing the tumor mass into a growing cell cycle appears to achieve better responses to radiation and chemotherapy.

Surgery may also include the placement of shunts into the brain. This relieves the pressure in the brain by using single direction catheters inserted in the ventricles of the brain to drain excess fluid into other body cavities, such as the right ventricle of the heart or the abdomen. If complete or partial resection is not possible and diagnosis is difficult by imaging techniques such as MRI, biopsies are performed on these inoperable tumors to provide more information for the potential implementation of other therapies.

2.2. Radiation therapy

Following surgery to remove as much tumor mass as possible or if the tumor is in a non-operable region of the brain, radiation treatment can be performed to kill residual tumor cells and to relieve symptoms associated with the disease. Normal brain tissue can tolerate up to 60 Gy of radiation per dose, yet this is below the threshold required to kill GB cells. Over the course of conventional radiation therapy, patients are exposed to 180–200 cGy per dose, 5 days a week for 6 weeks for a total of 5400–6000 cGy treatment. Exposure of radiation is confined to the tumor mass and 2 cm of surrounding tissue. In order to minimize the brain damage caused by high dosage and to catch the tumor cells in more stages of cell growth, hyper fractionated radiation therapy is an option. This method also uses a dose of 5400–6000 cGy radiation, but delivery is altered by giving more frequent fractions of a smaller dose over a smaller region of the brain. As with all radiation treatments, the side effects include swelling of the brain and the accumulation of dead cells, making it difficult to assess tumor reduction during routine scans.

In order to deliver more precise and higher doses of radiation therapy, stereotactic radiosurgery has been developed. The benefits of this method allow for the delivery of high-dose radiation to tumor masses <1.5 inches in diameter, while minimizing the exposure of normal brain tissue to radiation damage. Computer imaging is used to deliver high-energy doses to the precise tumor shape. This procedure can also be carried out on patients who already have had conventional radiation therapy and have been exposed to the maximum radiation dosage, yet still have residual tumor.

If the tumor is well-defined, surgically accessible, and no more than 5 cm in diameter, interstitial radiation therapy (or brachytherapy) can be used. This method uses radioactive pellets implanted within the tumor to kill the cancerous mass, minimizing the exposure of normal brain tissue to radioactivity.

Boron Neutron Capture Therapy (BNCT) uses thermal or epithermal neutron beams to activate a boron drug concentrated in the tumor cells. When exposed to the radiation, the boron becomes unstable, disintegrates, and induces cell death by releasing harmful radiation. Both components on their own are not harmful to normal brain tissue.

Several technologies have been developed to enhance the effect of radiation therapy or to minimize its size effects. Hyperthermia is defined as using heat to enhance the response of the tumor to radiation. Heat is provided by radiofrequency, microwaves, or ultrasound, requiring the even distribution of heat throughout the tumor mass in order to achieve therapeutic benefit. Radiosensitizers are drugs used to make the tumor mass more sensitive to radiation therapy. In order to be effective, the drugs must concentrate to the tumor mass. Examples of these drugs are BudR, IudR, hyperbaric oxygen, and tirapazamine. Radioprotectors (i.e., DFMO) are used to protect normal brain tissue from the effects of radiation (Table 2). Hyperthermia can also be used to enhance the beneficial effects of chemotherapy.

Table 2
Chemotherapeutic agents used for the treatment of brain tumors

Class	Type	Mode of action	Drug	Brain tumor type
Alkylating agents	Nitrosoureas	Cause DNA cross-links and, in some cases, carbamoylation of amino groups	BCNU CCNU ACNU MeCNU PCNU Fotemustine	Malignant glioma, brain metastases
	Platinum coordination	Inhibit DNA synthesis through intrastrand cross-links	Cisplatin Carboplatin Iproplatin	Malignant glioma, PNET, germ cell tumors Medulloblastoma
	Bis(cloroethyl) amines and mustard alkylators	Produce carbonium ions that react with electron-rich areas in susceptible molecules	Cyclophosphamide Ifosfamide Melphalan Nitrogen mustard	Malignant glioma, PNET, germ cell tumors, medulloblastoma
	Azirdine	Produce carbonium ions that react with electron-rich areas in susceptible molecules	Thiotepa	In bone marrow transplant programs for malignant glioma, medulloblastoma, other PNET
	Triazine	Forms diazo alkylating species	Dacarbazine Temozolomide	Malignant glioma, brain metastases
	Benzoquinone	Produces DNA strand break and DNA cross-links	Azirdinyl benzoquinone (AZQ)	Malignant glioma
Antimetabolites	Folic acid analogs	Inhibit dihydrofolate reductase, block thymidylate synthesis	Methotrexate	Medulloblastoma, PNET, PCNSL
	Pyrimidine analogs	Inhibit critical enzymes for nucleic acid synthesis, are incorporated into DNA and RNA	Fluorouracil Cytarabine	Malignant glioma, brain metastases PCNSL
	Purine analogs	Interfere with normal purine interconversion and thus, with RNA and DNA synthesis	Thioguanine Mercaptopurine	Malignant glioma
Natural products	Vinca alkaloids	Affect microtubular protein inhibit mitosis	Vincristine	Malignant glioma, PNET
	Podophyllotoxins	Topoisomerse II inhibitors, cause DNA breakage and arrest of cells in S phase	Etoposide (VP-16) Teniposide (VM-26)	Malignant glioma, PNET, germ cell tumors, brain metastases Medulloblastoma
	Antibiotics	Cause DNA strand scission topoisomerase II-dependent DNA cleavage, and intercalate with DNA double helix	Bleomycin Doxorubicin Idarubicin	Pediatric brain tumors Pediatric brain tumors, malignant meningioma
Miscellaneous	Urea analog	Inhibition of enzymatic conversion of ribonucleotides	Hydroxyurea	Malignant glioma, meningioma
	Methylhydrazine derivative	Mechanism not well known; affects DNA, RNA and protein synthesis, probably through azomethoxy reactive group	Procarbazine	Malignant glioma, PNET, PCNSL, medulloblastoma
	Halogenated hexitols	Act as an alkylating agent with effects on DNA, RNA	Dibromodulcitol	Medulloblastoma, ependymoma, brain metastases
	Polyamine inhibitors	Irreversible inhibition of ornitine decarboxylase	Difluoromethyl ornitine (DFMO)	Malignant glioma

2.3. Chemotherapy

The use of chemotherapy is now well established in the treatment of brain tumors. Chemotherapy prolongs survival, especially in patients with anaplastic gliomas, oligodendrogliomas, medulloblastoma, primitive neuroectodermal tumors (PNET), germ cell tumors, and primary CNS lymphoma (PCNSL). Glioblastoma tends to become chemoresistant. As is also the case with many systemic cancers, chemotherapy of brain tumors is not curative, and the goals of the treatment are mainly to control the growth of the tumor and to maintain good performance and quality of life for the patient for as long as possible (Burton & Prados, 2000; Beauchesne, 2002; Kleinberg et al., 2002; Tentori & Graziani, 2002; Trent et al., 2002; Watling & Cairncross, 2002).

Chemotherapy can be used as a primary therapy or an additional therapy following surgery and/or radiation therapy. The most common agents are the nitrosoureas (BCNU, CCNU), platinum-based drugs (cisplatin, cisplatinum, carboplatin), temozolomide, procarbazine, and natural-occurring compounds (taxol) (Table 2). The drawbacks to this

therapy are that not all tumors are responsive to chemotherapeutic drugs, and treatment causes damage to the bone marrow of the patient. Actively dividing cells are most susceptible to this form of treatment.

There have been several enhancements to chemotherapy treatments. Intracavity or interstitial therapy uses implanted catheters and polymer wafer implants to directly deliver chemotherapeutic agents into the tumor. There are also high-dose treatments, in which the drug is administered to the patient, followed by an antidote to overturn excess damage (i.e., methotrexate and leucovorin). Drugs with lower toxicities (i.e., temozolomide and phenylacetate) are also being developed that have decreased side effects. Receptor-mediated permeabilizers (RMP-7) that increase the permeability of the BBB to allow for the drugs to enter the brain have also been utilized to treat GB (Dean et al., 1999; Warren et al., 2001; Grant et al., 2002).

The major drawback with chemotherapy is the development of chemoresistant cells within the tumors and inadequate drug delivery methods. Sometimes the chemoresistance can be overcome by exposing the tumor to multiple types of antitumor drugs. Nevertheless, cells within the tumor can have varying sensitivities to the drugs, which, in turn, can produce resistant clones and eventual progression of the tumor. The failure of aggressive chemotherapy treatment to eradicate brain tumors is due in part to the presence of the BBB. The BBB separates the brain from blood to help regulate brain function and metabolism. It is composed of layers of cells consisting of capillary endothelial cells, astroglia, pericytes, and microglia in a basal lamina. The physical barrier depends largely on the endothelial cells being connected together with continuous tight junctions, with a high electrical resistance. Electrical resistance is responsible for the poor penetration of polar and ionic substances. In addition, different efflux pumps are present in the BBB, for example, P-glycoproteins, organic anion transporters, and multidrug resistance-associated proteins. Therefore, the BBB acts as a barrier for most hydrophilic substances and for larger lipophillic molecules. In addition, because of the presence of efflux pumps, not all small lipophilllic molecules are able to enter the brain, unless they have suitable physicochemical features. There is evidence that BBB in tumor sites may have higher permeability than exists in normal brain tissue, as the tumor causes subtle alterations in tight junctions.

The response of brain tumors to chemotherapy varies depending on tumor type, the feasibility and extent of surgical resection, the age of the patient, and general and neurological performance status. We will discuss briefly chemotherapy for malignant gliomas, as these tumors are the main focus of this review. Patients with anaplastic astrogliomas (AA) usually respond better to chemotherapy than patients with gliobastoma multiforme (GB) (Table 2). Nitrosourea-based drug combinations showed increased antitumor efficacy when compared with single agent BCNU. Significant prolongation of median survival was demonstrated by Levin (1999) for AA patients on the combination of procarbazine, CCNU, vincristine (PCV; median 157 weeks) compared with a group on BCNU (median 82 weeks). Studies of radiation therapy (XRT) with bromodeoxyuridine followed by PCV showed a median survival of 4 years (Levin et al., 1995b; Prados et al., 1998). In a non-randomized trial of oral 6-thioguanine prior to BCNU, comparable results were reported for AA patients, who had a median survival of over 4 years (Prados et al., 1998). The results of a phase II study of temozolomide versus procarbazine in patients with GB at first relapse have been reported (Yung et al., 2000). The primary objectives of this study were to determine progression-free survival at 6 months and safety in adult patients who failed conventional treatment. Overall, progression-free survival significantly improved with temozolomide, and importantly, it had an acceptable safety profile; most adverse events were mild or moderate in severity (Yung et al., 2000).

Chemotherapy is generally used as adjuvant following surgery and XRT, or as palliative treatment for patients who failed prior therapy (Levin et al., 1985; Shapiro et al., 1989; Recht et al., 1990; Dropcho et al., 1992; Vega et al., 1992; Watne et al., 1992; Yung et al., 1992). Chemotherapy can also be initiated in conjunction with XRT for a dual effect of radiosensitization and drug cytotoxicity (Levin et al., 1995a, 1995b; Hellman et al., 1998). BCNU, cisplatin (i.v. or i.a.), and carboplatin are presently used together with XRT in clinical trials. In spite of new chemotherapeutic agents, new drug combinations, and new delivery methods, the success of this therapeutic modality to treat glioblastoma multiforme is limited (Hellman et al., 1998). Promising survival results for patients with newly diagnosed glioblastoma multiforme has been reported recently, after they were treated with concomitant radiation plus temozolomide, followed by adjuvant temozolomide (Stupp et al., 2002). The prevention of irradiation-induced glioma cell invasion by temozolomide involves caspase-3 activity and cleavage of focal adhesion kinase (Wick et al., 2002).

Therefore, for GB in particular, there is a great need to develop novel therapies or to enhance the tumor-specific cytotoxic effects of current treatment regimens. Relapse in these patients occurs in $\sim$80% of cases in the form of continuous growth within 2–3 cm from the margin of the original lesion, with only occasional intraventricular spread or dissemination along the craniospinal axis leading to secondary tumors. These patients are, therefore, good candidates for the testing of new treatment modalities.

3. Novel therapies for brain tumors

3.1. Gene therapy

Gene therapy can be defined as the use of nucleic acids as drugs. As such, it can modify the genetic make-up of the target cells, which is not possible to achieve with any other

therapeutic modality. Gene therapy is a very attractive approach for the treatment of cancer, and the particular focus of this review is how we can harness the power of various gene therapy approaches to treat brain tumors. In order to deliver the therapeutic gene(s) to the tumor mass, we need to use delivery or vector systems that can be subdivided into two main groups, i.e., viral and non-viral delivery vectors.

The optimal gene delivery system would be a vector capable of encoding for large DNA inserts and efficiently transducing the target tissue, in which expression of the therapeutic gene would be of sufficient duration and selectively targeted to the tumoral cells. In addition, the vector should be easily manufactured ideally; be able to be delivered systemically; and be non-cytotoxic, preferably allowing repeated administration.

Viral vector systems have been chosen for the majority of gene therapy clinical trials, with the most popular being retrovirus, adenovirus (Ad), adeno-associated virus (AAV), and herpes simplex virus (HSV) vectors.

3.1.1. Viral gene delivery

The usefulness of viruses as gene delivery vectors centers on their natural ability to enter and mediate expression of transgenes in infected host cells. In order to ensure that the viruses used as vectors are safe and cannot rampantly infect the host organism, it is important to produce viral vectors that are replication defective. The removal of several viral functions involved in replication and, therefore, viral spread also creates physical space within the virus genome for the insertion of therapeutic genes. The majority of gene therapy protocols that are currently approved for clinical trials are based on viral delivery systems. The main viral vector systems currently being developed are described below.

3.1.1.1. Adenovirus vectors. In humans, adenoviruses (Ads) cause benign infections, such as respiratory tract infections, keratoconjunctivitis, and gastroenteritis. Ads can infect a wide variety of cell types with high efficiency and no risk of insertional mutagenesis, making them attractive vectors for gene therapy.

Ads are large 60–90 nm diameter, non-enveloped particles with a double-stranded DNA genome of 36 kb (Horne et al., 1975). Protecting the genome is the symmetrical protein icosahedron coat comprised of 252 capsomeres (240 hexons and 12 pentons). The penton capsomere contains a penton base and a trimeric fiber protein (ending in a globular knob protein) that projects from each vertex of the virus (Fig. 1).

The adenoviral genome enters dividing, as well as non-dividing, cells by receptor-mediated endocytosis. Infection is initiated by the binding of the fiber protein to the coxsackie virus and adenovirus receptor (CAR), which is expressed at the cell surface (Bergelson et al., 1997, 1998). Interaction of the fiber protein with other cellular receptors such as the MHC class I molecule has also been implicated (Hong et al., 1997). Entry into the cell is then further mediated by the penton base of the adenoviral capsid, which engages integrin receptor domains ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) on the cell surface, resulting in internalization of the virus into an endosome (Wickham et al., 1993). Once within the endosome, a decrease in the internal pH causes conformational changes of the viral coat proteins causing viral escape into the cytoplasm. The capsid then translocates to the nuclear membrane, triggering dissociation of the viral coat proteins to expose the viral DNA. The viral DNA can then enter the nucleus to undergo transcription and replication episomally without the requirement for cell division.

Expression from the linear, double-stranded genome of the Ad is divided into two phases of gene expression divided by adenoviral DNA replication. There are four distinct early regions (E1–E4) and an alternatively spliced major late-region consisting of five expression regions (L1–L5). At the terminal sequence of each genome strand are inverted repeats (ITRs) and a 55-kDa terminal protein covalently attached to the 5′ end (Fig. 1). The E1 genes are transcribed first and their proteins then regulate most other viral transcription units in a cascade. E1A proteins modulate the host cell cycle so that it enters into S phase to provide an optimal environment for viral replication, while E1B blocks host cell apoptosis by binding to the tumor suppressor gene p53. E2a encodes a protein with DNA binding and regulatory functions, and E2b encodes a DNA polymerase and terminal-binding protein. E3 proteins are involved in viral mechanisms to evade the host immune system, and E4 encodes multifunctional proteins for viral transcriptional control, DNA replication, and shut-off of host cell protein expression. The major late-region genes encode the Ad structural components (including the capsid proteins), which are assembled to package the DNA, producing infectious progeny. These viral particles accumulate in the nucleus until the cell is lysed, leading to virion release.

Of the 50 or more adenovirus serotypes, Ad 2 and 5 have predominantly been used for gene therapy. First-generation Ad vectors (rAds) are rendered replication defective by deletion of the E1 gene region, which allows the insertion of foreign DNA (up to 8 kb). To allow the insertion of larger therapeutic transgene cassettes, the E3 region may also be deleted. E1/E3-deleted rAds are generated by homologous recombination between a shuttle plasmid and a rescue plasmid. The shuttle plasmid provides the expression cassette (encoding the promoter, therapeutic gene, and polyadenylation signal) with flanking homology from either side of the Ad E1a gene region. The Ad genome plasmid contains the entire Ad5 genome with prokaryotic "stuffer" DNA inserted into the E1a gene, making this plasmid too large to be packaged into a capsid. The plasmids are co-transfected into 293 cells that have been stably transfected to provide, in trans, E1 gene complementation (Graham et al., 1977). Homologous recombination between the plasmids results in the expression cassette replacing both the E1a

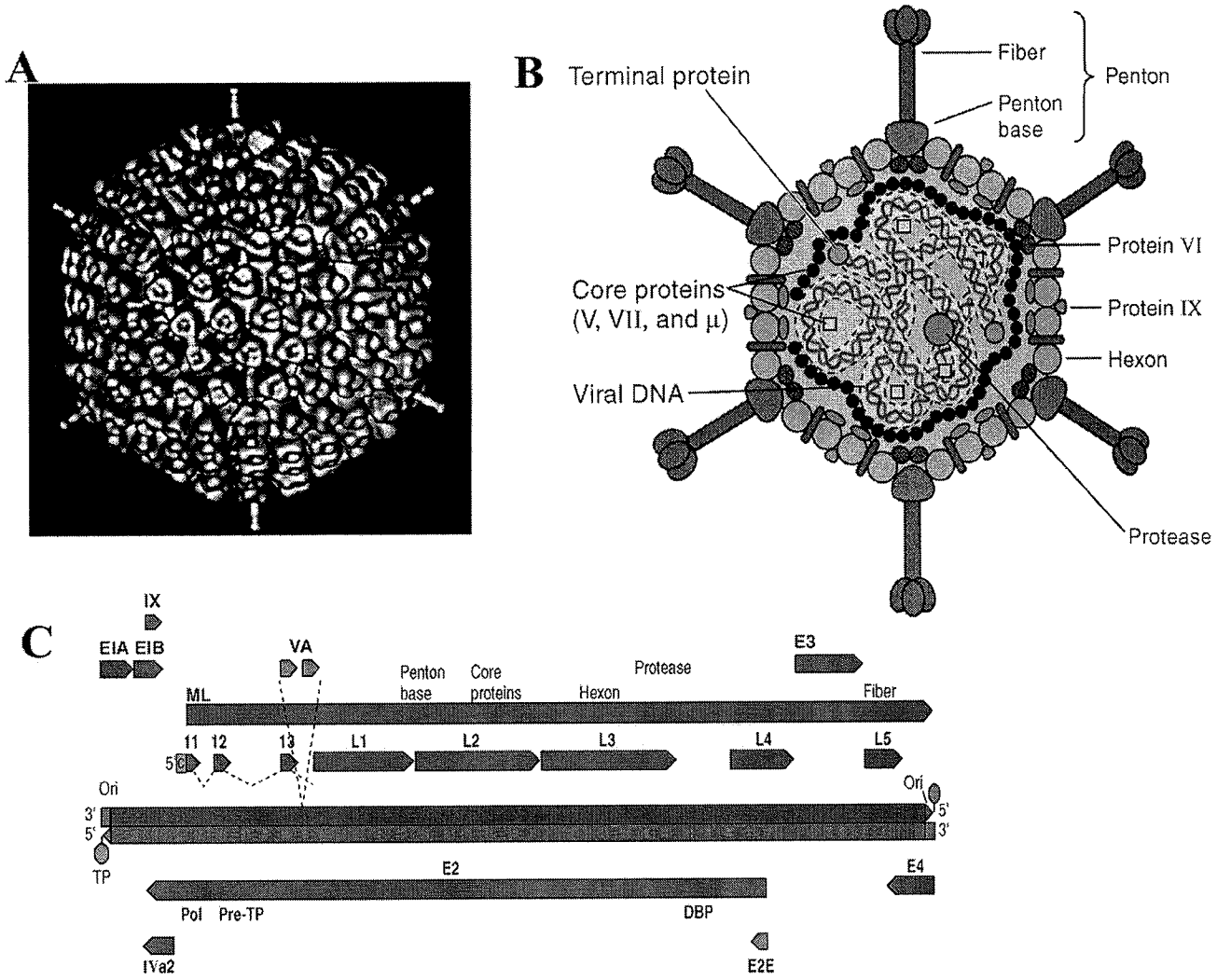

Fig. 1. Adenovirus virion. **A**: Three-dimensional image of the intact adenovirus particle viewed along an icosahedral 3-fold axis. **B**: Schematic representation of an adenovirus particle based on the current understanding of its polypeptide constituents and genome (Flint et al., 2000). **C**. Adenoviral genome and transcription map. Genome is 100 map units (mu) in length (1 mu = 360 bp). Early primary mRNA transcripts are designated in bold. Certain polypeptides are identified by conventional numbering system (roman numerals) (Flint et al., 2000).

region and intervening stuffer DNA. As a result, the recombinant DNA size is sufficiently reduced for packaging and generating an E1-deleted recombinant virus containing the gene of interest. RAd vectors are propagated in 293 cells and can be produced to titres as high as 10^{12} plaque forming units (pfu)/mL.

Transgene expression from first-generation rAd vectors tends to be transient in vivo (Yang et al., 1994b). However, our group and others have shown persistent (6–12 months) transgene expression in the brain (Geddes et al., 1997; Dewey et al., 1999; Thomas et al., 2000). Short-term expression in the periphery results from 90% of the administered vector being degraded in the liver by a non-immune-mediated mechanism following intravenous delivery (Worgall et al., 1997). However, the immune response elicited by these vectors in vivo is probably one of their major drawbacks. Most adults have had prior exposure to wild-type Ads and, therefore, possess anti-Ad antibodies (Abs). Studies show that 55% of adults have Ads capable of neutralizing the infection of Ad5, the most widely used serotype for rAd vector construction (Chirmule et al., 1999). Upon rAd vector administration in the periphery, an MHC class I immune response is observed, characterized by the activation of $CD8^+$ cytotoxic T lymphocytes (CTLs) to eliminate virus-infected cells and $CD4^+$ cells, which secrete IFN-γ that in turn results in anti-Ad antibody production (Yang et al., 1994a; Yang & Wilson, 1995). This immune response possibly contributes to limiting the duration of transgene expression and antibody production precludes repeated administration of rAd. Work from our laboratory has shown that circulating anti-Ad antibodies would limit duration of therapeutic transgene expression from first-generation Ad in the brain, without affecting expression from gutless Ad. These results indicate that using gutless, it should be

possible to readminister the vectors and/or allow very long-term transgene expression, even in the presence of circulating antibodies (Thomas et al., 2000, 2001b). For gene therapy applications to treat brain disease such cancer, these immune responses are somewhat different, in that transgene expression mediated by Ads in the CNS is long lived, up to 1 year (Thomas et al., 2000); inflammatory responses are only transient, dose-dependent, and they do not effect transgene expression in a deleterious way (Thomas et al., 2001a).

Great efforts have been made to develop second- and third-generation vectors with reduced immunogenicity. Through the mutation or removal of other Ad genes, such as the E2, E3, and E4 gene regions from the viral genome, the expression of many viral proteins can be reduced, thereby reducing the host immune response (Englehardt et al., 1994a, 1994b; Yang et al., 1994a; Dedieu et al., 1997; Wang et al., 2000). However, in all of these vectors, some viral proteins that are still expressed at a very low level are presented to the immune system.

Later generations of Ad vectors are deleted for all viral genes and contain only the ITRs and the packaging signal of wild-type Ad. These vectors are termed "gutless", "helper-dependent" (H-D), or "high capacity" (HC) vectors, as they have the potential to accommodate 35 kb of foreign DNA. To generate HC rAd, a helper virus is required providing all the essential viral genes in trans. To purify the HC vector, LoxP sites flank the viral packaging signal of the Ad helper. Both HC and helper viruses are propagated in 293 cells expressing Cre recombinase, which excises the helper packaging signal so that only the HC vector can be packaged, producing a preparation with as little as 0.01% helper virus contamination (Parks et al., 1996). A similar system has been created by establishing 293 cells expressing a FLPe-recombinase and a helper virus containing a packaging signal flanked with FLPe-recombinase target (FRT) sites (Umana et al., 2001; Lowenstein, 2002) (Fig. 2). In contrast to first-generation vectors, gutless vectors can promote stable transgene expression in peripheral organs, including the liver and also in the CNS with reduced immunogenicity (Thomas et al., 2000; Zou et al., 2000, 2001). Peripheral administration of these vectors also allows repeated administration of the virus without increased vector-mediated toxicity or loss of therapeutic response (O'Neal et al., 2000). Our group demonstrated that gutless Ad vectors succeeded in maintaining long-term transgene expression in the brain, even in animals previously exposed to Ad and presenting an active peripheral immunization to Ad that completely eliminated expression from first-generation vectors (Thomas et al., 2000, 2001b).

For the delivery of therapeutic genes for the long-term treatment of neurological disorders, first-generation rAd vectors may prove very useful. These vectors do have an appeal specifically for cancer gene therapy. For most cancer treatment strategies, we would wish to kill the tumor cells, and transient gene expression would be satisfactory for this purpose. The immune responses, which could be elicited by Ad infection, may also have benefits for cancer treatment.

Further advances in rAd vectors for cancer gene therapy have used conditional (or restricted) replicative rAd systems. The ONYX-015 vector is an E1B 55-kDa gene-defective Ad that restricts its viral replication to p53-deficient tumor cells, inducing specifically their lysis (Bischoff et al., 1996). Furthermore, the efficacy of the tumor-specific lysis of ONYX-015 is increased when it is used in conjunction with chemotherapy agents, cisplatin, and 5-fluorouracil (Heise et al., 1997).

A second tumor-selective replicative rAd has also been developed specifically to kill glioma cells or other cancer cells with a disrupted retinoblastoma (Rb) pathway. This vector, Delta24, has a 24-bp deletion in the E1A region responsible for binding Rb protein and can, therefore, specifically lyse Rb defective glioma cells (Fueyo et al., 2000). As Rb mutations are the most common genetic mutations of anaplastic astrocytoma, this vector may prove as useful as the ONYX vector.

3.1.1.2. Adeno-associated virus vectors. Adeno-associated viruses (AAVs) are small single-stranded DNA viruses of the *parvovirus* family. They are non-pathogenic in humans and non-autonomous, requiring another virus, such as Ad, HSV, or vaccinia virus, to replicate (Atchison et al., 1965; Schlehofer et al., 1986). AAVs are capable of infecting a large variety of both dividing and non-dividing cells since they interact with the host cell through heparin sulfate proteoglycans and integrins (Summerford & Samulski, 1998; Summerford et al., 1999). AAVs have two distinct life-cycle phases. In the absence of a helper virus, AAV genome integrates into a specific locus (AAVS1) of the host genome (q arm of chromosome 19 in human cells) at high frequency (Kotin et al., 1990; Linden et al., 1996). Following integration, they remain latent, but in the presence of a helper, the lytic phase of the virus is induced. In the presence of the Ad helper, the early Ad genes are expressed and lead to active AAV replication.

The AAV is a small icosahedral particle (20–24 nm diameter) and is non-enveloped. Among the six known serotypes, AAV2 has been the most extensively characterized. The linear single-stranded DNA genome of only 4680 bases is flanked by 145 bp inverted terminal repeats (ITRs) and encodes for two open reading frames: *rep*, coding for four main proteins that control AAV replication, structural gene transcription, and integration into the host genome and *cap*, which encodes for structural proteins that form the viral capsid (Rose et al., 1969; Srivastava et al., 1983). The first 125 bp of the ITRs are palindromic and form a hairpin structure, enabling the initiation of replication. The ITRs are also required for encapsidation and integration into a specific locus within the chromosome 19.

Recombinant replication-defective AAV (rAAV) vectors for gene therapy lack *rep* and *cap* sequences. They can be produced by cotransfecting two plasmids into permissive

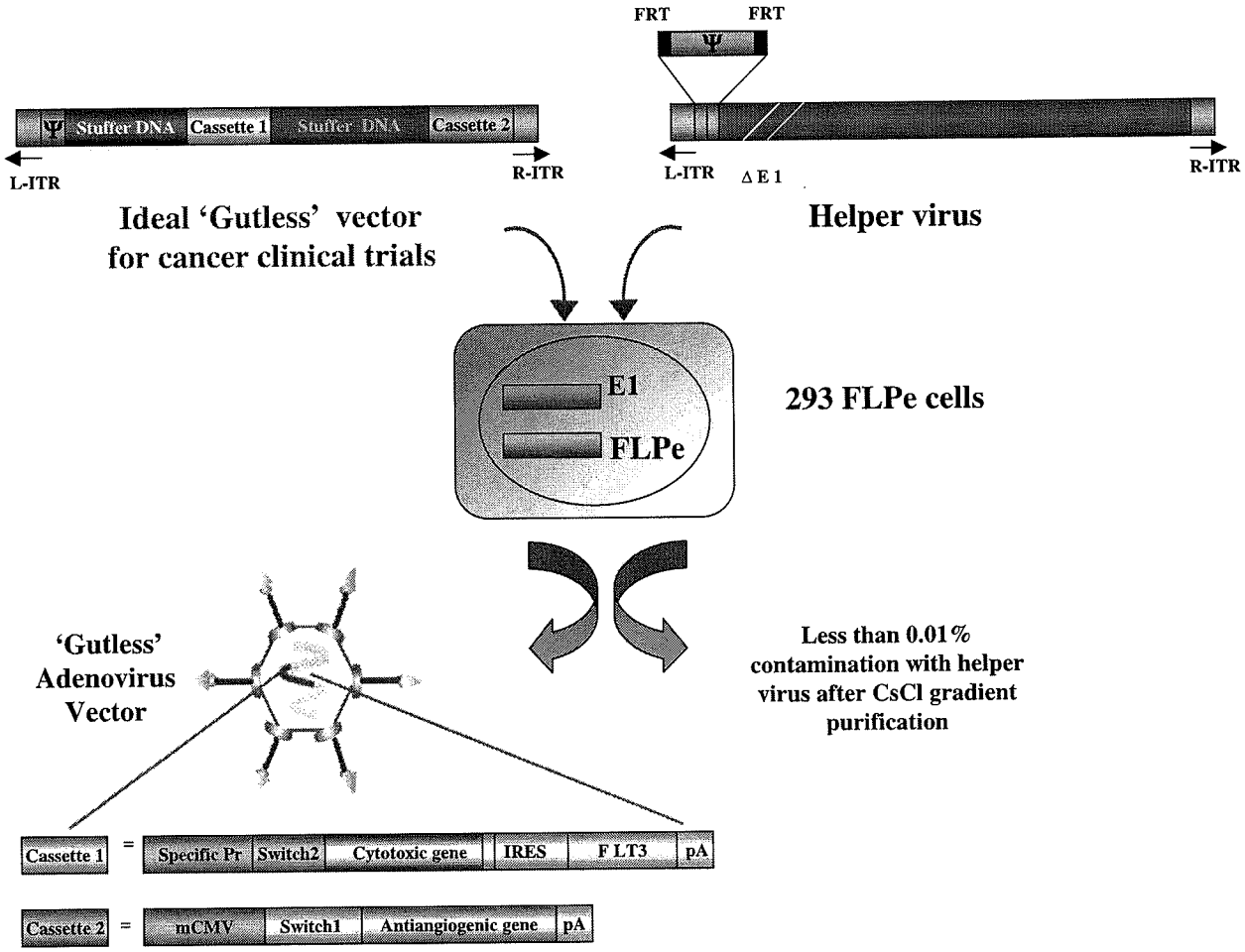

Fig. 2. Ideal gene therapy adenoviral vector for effective treatment of gliomas. Diagram of an ideal gene delivery system for gene therapy strategies using the 'gutless' adenoviral vector to produce a regulatable expression of a cytototoxic, anti-angiogenic, and an immune stimulatory gene product, i.e., flt3 L. Flt3L is a potent hematopoyetic growth factor that increases dendritic cell infiltration in the tumor region after its expression from an adenoviral vector. The packaging signal of the helper virus, flanked by FRT sites, is excised after passing in 293 cells expressing Flpe recombinase. The expression of the two therapeutic cassettes can be regulated independently because they are under the control of two different regulatable switches. Furthermore, the expression of the cytotoxic gene is under the control of a cell type specific promoter(Pr) to achieve a cytotoxic effect only in tumor cells. flt3L, fms-like tyrosine kinase 3 ligand; IRES, internal ribosome entry site; LITR, left-inverted terminal repeat; mCMV, murine-early cytomegalovirus virus promoter; RITR, right-inverted terminal repeat.

293 cells (Samulski et al., 1989). The first plasmid contains the transgene cassette flanked with ITRs, and the second plasmid provides *rep* and *cap* ORFs. This system requires the infection with a helper virus to activate the replication. However, this method is prone to contamination with Ad and wild-type AAV, and produces a low yield. A newer method based on a three-plasmid transfection system has been developed (Xiao et al., 1998). The first two plasmids are identical as the one described above and the third encodes a mini Ad genome with all of the Ad structural genes removed, but containing all the sequences necessary for the replication. This methods leads to only rAAV vectors; however, it is very laborious for large-scale preparations. Therefore, alternative strategies, including hybrid vectors or packaging cell lines, have been explored. Because the wild-type genome is only 4.7 kb, the total packaging capacity of rAAV cannot greatly exceed this, restricting the amount of foreign DNA that can be introduced. Here again, some groups have investigated the possibility of forming concatemers allowing at least to double the size of the transgene. Another characteristic of rAAV is that they have been shown to remain episomal in certain cell types. However, efficient long-term rAAV-mediated gene transfer has been demonstrated for up to 10 months in the periphery, with no evidence of a cellular immune response (Rivera et al., 1999). It recently has been reported that AAV vectors can integrate as vector provirus, and this integration is associated with chromosomal deletions and other rearrangements that are frequently located on chromosome 19 (not at the wild-type AAV integration site) (Miller et al., 2002). This phenomenon has important implications for the use of these vectors in gene therapy applications, as it might influence both transgene and endogenous cellular gene expression.

The use of rAAV vectors for cancer gene therapy is still at the level of preclinical study. Recombinant AAV vectors encoding for HSV-TK achieved tumor regression of human gliomas transplanted into the brain of nude mice (Mizuno et al., 1998).

3.1.1.3. Herpes simplex virus vectors. Two gene transfer strategies using Herpes simplex virus type 1 (HSV-1) have been used, the first one is based on recombinant vectors, and the other is using amplicons. HSV-1 is a ubiquitous human pathogen possessing a large linear double-stranded DNA genome of ~150 kb, encoding for 70–80 genes. Like Ad and AAV, HSV-1 has naturally a broad host range due to the ability of the HSV envelope glycoproteins (gB and gC) to bind to the extracellular heparin sulphate moieties of cell surface proteoglycan molecules found in all cell membranes (WuDunn & Spear, 1989). The envelope protein gD is then required for internalization of the virus. HSV-1 can infect both dividing and quiescent cells, where it can persist for a long time in a non-integrated state, but infection of non-neuronal cell types results in host mRNA degradation and shut off of protein synthesis (Read & Frenkel, 1983). Contained within the matrix of the HSV particle is the tegument protein VP16 that is carried into the cell upon infection. VP16 induces a viral gene cascade, beginning with the immediate expression of the HSV immediate early (IE) genes, ICP0, -4, -6, -22, and -27. These genes then instigate the expression of the early and late genes required for replication and encapsidation.

Much of the large genome of HSV is redundant, enabling large deletions to be made, allowing as much as 50 kb of heterologous DNA to be accommodated, without detrimentally affecting viral growth (Wang et al., 2000). The first HSV vectors were rendered replication defective by mutation of the IE gene ICP4, with the creation of an ICP4-expressing complementary cell line to allow the production of these vectors. Unfortunately, these initial HSV vectors were toxic due to a potent immune response against the IE genes of the virus. Therefore, further vectors deficient for other, or even all, IE viral genes have been developed (Glorioso et al., 1995). Paradoxically, the genetic manipulations required to eliminate toxicity and allow the genome to persist in cells for longer periods of time have been shown to dramatically lower the level of transgene expression (Samaniego et al., 1998).

An alternative strategy utilising amplicons to generate HSV vectors can be used to generate a vector expressing none of the IE genes. Amplicons are plasmid vectors containing HSV packaging and replication signals, which enable them to replicate and be packaged in the presence of a helper virus providing the missing HSV gene products in trans. As HSV DNA replication occurs by a rolling circle mechanism, the resulting amplicon vector consists of the husk of HSV (envelope, tegument and capsid), with multiple copies of the recombinant DNA plasmid carrying the therapeutic gene, up to the upper limit for packaging of the HSV virion (Frenkel et al., 1994). First-generation amplicon systems were highly contaminated with helper virus, but newer methods of HSV production have generated helper-free preparations (Geller et al., 1997). There are evident advantages for the use of HSV vectors: notably, their large insert capacity as a viral vector that can carry multiple genes may best attack the complex mechanisms of cancer. They also have a wide host range and the ability to establish latency in neurons, leading to long-term transgene expression.

Defective HSV-mediated gene transfer of tissue inhibitor of metalloproteinases-2 (TIMP-2) has been used to inhibit the invasive properties of malignant glioma cells in vitro (Hoshi et al., 2000). Replicative HSV vectors are also being investigated, including the replicative mutant HSV vector hrR3, which encodes for an intact thymidine kinase (TK) gene and can kill cells via replication and cell lysis, or by sensitizing them to ganciclovir. To increase specificity for tumor cells, hrR3 has a mutated ribonucleotide reductase gene and replicates selectively in cells with high levels of endogenous ribonucleotide reductase. Actively dividing cells such as tumor cells have high levels of endogenous ribonucleotide reductase for synthesis of DNA precursors. HrR3 has been proposed as a particularly good vector for the treatment of brain tumors because it can replicate in dividing gliosarcoma tumor cells, causing their lysis, but cannot replicate in post-mitotic neural cells (Boviatsis et al., 1994; Mineta et al., 1994; Spear et al., 2000).

Despite all this, there still remain challenges with virus toxicity, which has hampered the use of HSV vectors for gene therapy and precluded their use beyond animal models.

3.1.1.4. Retroviral vectors. The majority of human gene transfer clinical protocols approved to date use retrovirus vectors, ~34%; followed by adenovirus vectors, 27%; lipofection, 12%; naked plasmid DNA, 11%; Pox virus, 6.1%; adeno-associated virus, 2.4%; RNA transfer, 0.9%; and herpes simplex virus, 0.8%. Retroviruses have a diploid RNA genome of 8–11 kb, packaged into a virion and surrounded by a lipid envelope, comprised from both host cell membrane and viral proteins. The genome is flanked by long terminal repeats (LTRs) which include promoter/enhancer regions and sequences involved with integration (LTRs) and encodes for a packaging signal (ψ), structural capsid proteins (*gag*), a viral protease (*pro*), integrase, and viral reverse transcriptase (*pol*) and envelope glycoproteins (*env*). Different retroviruses bind different cellular receptors. The most commonly used retroviral vectors are based on the Moloney murine leukemia virus (Mo-MLV), which binds to the transmembrane phosphate transporter RAM-1. The retroviral envelope fuses with the host cell membrane, releasing the virion capsid core into the cytoplasm. Once inside the cytoplasm, the RNA genome must be reverse transcribed into double-stranded DNA using the viral reverse transcriptase. This complementary DNA sequence complex with the viral integrase protein and is transported

to the nuclear membrane. During mitosis, the membrane of the nucleus is disrupted, allowing the complex to enter and integrate into a host cell chromosome. The integrated virus is then able to use the host cell machinery for viral gene expression.

Retroviral vectors are rendered replication defective by the deletion of the genes *gag*, *pol*, and *env*. Vector production then requires a viral packaging cell line to provide these deleted genes in trans. The packaging cell line is optimally constructed by stably inserting the deleted viral genes *gag*, *pol*, and *env* into the packaging cell genome, such that each gene will reside on different chromosomes. This approach ensures that recombination of these genes is highly unlikely, minimising the possibility of the generation of replication-competent retroviruses, and produces a vector in which the only viral sequences are ψ and the LTRs.

Unfortunately, the integration of retroviral vectors is random and limited to dividing cells. The insert capacity of these vectors is 8 kb, and at present, only moderate retrovirus titers of 10^5-10^7 plaque-forming units (pfu)/mL can be produced. VSV-G pseudotyping of these vectors increases Mo-MLV retrovirus titers up to 10^8 pfu/mL (Pakkanen et al., 1999). In addition, complement proteins present in human sera can inactivate retroviruses (Rollins et al., 1996), and transgene expression can also be reduced by inflammatory interferons, which, as the retroviral genome integrates into the host genome, are thought to inactivate the viral LTR promoters. However, with stable integration of this vector into host DNA and low immunogenicity, because of the removal of all genes encoding for viral proteins, long-term therapeutic transgene expression of over 2 years has been achieved in humans (Bordignon et al., 1995).

Retrovirus vectors, based upon the Mo-MLV, are limited in their application to cancer gene therapy by their inability to infect non-dividing cells. While cancer cells are often undergoing high rates of cell division, it has been shown that in some malignancies, including the majority of brain tumors, especially within the internal areas of the tumor mass, the cells are not actively dividing. In addition, many applications of retroviral vectors in treating cancer would require a large number of viral particles, and because retroviruses are impossible to concentrate to high titers, it is often the case that grafts of vector-producing cells must be inserted into the tumor itself. This is a more complicated procedure than simply injecting a concentrated solution of virus, and poses additional immunological problems, leading to graft rejection. Despite this, more clinical trials using retrovirus have been approved than those using any other vector.

Far greater transduction efficiencies are being achieved with the more recent lentiviral vectors. Lentiviruses are a subclass of retroviruses that contain additional viral proteins. Two of these virion proteins, matrix (MA) and virion protein R (VPR), are used to transport the cDNA/integrase complex (also called pre-integration complex) across the nuclear membrane in the absence of mitosis. This allows lentiviruses to infect both proliferating and non-proliferating cells. The lentiviral vectors used are derived from the human immunodeficiency virus (HIV), but are pseudotyped by using the envelope glycoproteins of other viruses such as the vesicular stomatitis virus (VSV-G). The cell line 293 is used to generate these vectors using a 3-plasmid co-transfection system. Three separate plasmids encode for the pseudotype *env* gene, the transgene cassette, and a packaging construct, respectively, supplying the structural and regulatory genes in trans (Naldini et al., 1996).

These vectors have all the desirable properties of the early Mo-MLV-based vectors, but can infect both dividing and quiescent cells and induce a limited inflammatory response (Blomer et al., 1997). Lentiviruses have been used very successfully to infect and transduce the CNS, with almost negligible inflammatory responses (Deglon & Aebischer, 2002; Hsich et al., 2002; Stein & Davidson, 2002). Lentiviral vectors are currently being evaluated for safety, with a view to removing some of the non-essential regulatory genes to gain clinical approval. If the issues with production and safety can be overcome and higher titers can be achieved, these vectors hold great promise (Ailles & Naldini, 2002; De Palma & Naldini, 2002; Galimi & Verma, 2002; Huentelman et al., 2002).

3.1.1.5. Other viruses. Many other viruses have been tested as vectors for gene therapy: minute virus (Corsini et al., 1997), Semliki Forest virus (Duncan et al., 1999), Sindbis virus (Iijima et al., 1999), measles virus (Ehrengruber et al., 2001), Epstein-Barr virus (Tanaka et al., 1999), influenza virus (Neumann et al., 2000), and vaccinia virus (Mastrangelo et al., 2000). However, the work done with these viruses is at its infancy with respect to their use for the implementation of gene therapy clinical trials.

3.1.1.6. Hybrids. Several groups have been developing hybrids vectors, such as Ad/AAV (Recchia et al., 1999), Ad/retrovirus (Caplen et al., 1999; Duisit et al., 1999), retrovirus/vaccinia virus (Holzer et al., 1999), or hem agglutinating virus of Japan (HVJ)/liposomes (Kaneda et al., 1999), with the aim of combining the most attractive properties of each vector (Reynolds et al., 1999).

3.1.1.7. Targeted viral vectors. Prior to the discovery of the mechanisms by which rAd vectors infect host cells, tissue-specific expression was achievable only by direct delivery of the vector to the target area, for example, intratumoral injection (Dewey et al., 1999), or by using specific cellular promoters and enhancers (Smith-Arica et al., 2000, 2001). The selective cell killing of glioma cells was achieved using the astrocyte-specific promoter glial fibrillary acidic promoter (GFAP) to drive expression of the HSV-TK gene (Vandier et al., 1998).

Following the identification of CAR, to which the adenoviral fiber initially attaches, and also of the integrin family of cell surface heterodimers, to which the penton

base protein binds allowing internalization, methods of targeting adenoviral vectors are being developed. This also allows these vectors to deliver genes to tissues that are normally uninfectable by Ad, due to the low-level expression of CAR. Specific-tissue targeting will alleviate some of the safety concerns that have arisen surrounding the use of rAd vectors in human clinical trials, in addition to reducing the clearance of the vector by the liver, an organ that is easily transduced by Ad.

Genetic retargeting through modification of the fiber knob has been successfully achieved by different ways. The knob domain of classic rAds (based on the Ad type 5) was replaced by the knob domain of another serotype, such as type 3 (Stevenson et al., 1995, 1997; Krasnykh et al., 1996) or type 7 (Gall et al., 1996). Bispecific antibodies were developed to cross-link the fiber to cell-specific receptors (Douglas et al., 1996). Peptide motifs were attached to the C-terminus of the fiber knob (Wickham et al., 1995, 1997) or genetically inserted into the HI or DG loop of the fiber knob (Dimitriev et al., 1998; Krasnykh et al., 1998; Kamijo et al., 1999; Kirby et al., 1999). Modification of the C-terminus of the fiber of the conditionally replicative vector ONYX has also shown dramatically increased transduction and selective killing of glioma cells (Shinoura et al., 1999).

As an alternative to the modification of the fiber knob, the strategy of modifying the penton base has also been validated. The penton base was modified by insertion of a peptide (FLAG), which was then recognized by a specific antibody. By coupling the anti-FLAG Ab to another Ab recognizing exclusively a specific cell type receptor, this bispecific Ab was able to retarget Ad internalisation (Wickham et al., 1996).

Finally, another Ad component, the hexon, has also been modified by peptide insertion, illustrating another knob-independent entry pathway (Vigne et al., 1999). Recently, two groups have described new techniques to redirect Ad infection. Smith et al. (1999) covalently linked the Ad to biotin molecules and then biotinylated ligand were cross-linked through an avidin bridge. Romanczuk et al. (1999) have also used a bifunctional molecule, the polyethylene glycol (PEG), to retarget rAds.

To enhance gene transfer by rAd vectors to tumors, vectors have been designed that bind to receptors that are preferentially up-regulated in these tissues. Epidermal growth factor receptor (EGFR) retargeting of a rAd vector for the treatment of head and neck squamous cell carcinoma, using a bispecific antibody recognizing the knob domain of rAd as well as EGFR, has achieved increased specificity and transduction efficiency in vivo (Blackwell et al., 1999).

Retrovirus retargeting has also been addressed by using cross-linking Abs (Goud et al., 1988; Roux et al., 1989; Etienne-Julan et al., 1992), chemical coupling (Neda et al., 1991), or by modifying the envelope proteins (Russell et al., 1993; Kasahara et al., 1994). However, none of these techniques were really efficient. Finally, the best attempts have been obtained after targeting chimeric viruses in a two-step strategy (Nilson et al., 1996). The N-terminal envelope glycoprotein has been engineered with a ligand attached through a cleavable linker. The virus attaches the target cell through the modified ligand, and then the linker is cleaved by a specific enzyme, exposing the endogeneous retroviral binding sequence, thus allowing entry into the cell. Some targeting experiments using bispecific Abs have also been performed with AAV vectors (Bartlett et al., 1999) or Sindbis virus (Ohno et al., 1997).

3.1.2. Non-viral vectors

Although in the field of gene therapy viral vectors are more popular, many non-viral vectors are currently being developed. These include naked DNA, liposomes, and DNA complexed to protein/polymers.

In order to enter into host cells, *naked DNA* requires physical modification, such as calcium phosphate precipitation, DEAE-Dextran/chloroquine permeabilization, electroporation, heat shock, or intracellular microinjection. Unfortunately, most of these techniques are not adapted for in vivo gene delivery; therefore, direct injection and particle bombardment, also called "gene gun," are the most commonly used procedures for plasmid delivery. Mouse skeletal muscle and myocardial tissue have been shown to be highly receptive to plasmid (p) DNA transfection with expression of transgene persisting for longer than 2 months (Wolff et al., 1990). Numerous other tissues in vivo have been shown to be susceptible to pDNA-mediated transfection in vivo, including brain (Schwartz et al., 1996), although the efficacy is very low. Despite the great advantage of avoiding immune response against a carrier and not requiring any extra engineering/producing techniques, the use of naked DNA has been restricted because of its poor transduction efficiency. It has been used, however, in phase I/II clinical trial for immunotherapy of prostate or colon cancer, addressing the safety of the vaccine after repeated intradermal injections in humans (Mincheff et al., 2000, 2001).

Plasmid DNA *complexed with cationic lipids* to form liposomes has been shown to be highly successful in transfecting cell lines, which could be used for ex-vivo gene therapy approaches. Numerous cationic lipid compounds have been developed, some that spontaneously form liposomes (e.g., DOGS and DOTAP), whilst others require a neutral helper lipids to form stable lipid bilayers (e.g., DOTMA) (Gao & Huang, 1995). Many therapeutic genes, including Herpes simplex virus 1 thymidine kinase (HSV-1 TK), have been successfully delivered into tumors, including glioma, using cationic liposomes with favorable results (Yoshida et al., 2001). These successes have led to the development of clinical trials using cationic liposome mediated gene therapy for cancer. A phase I immunotherapy study using cationic liposomes to deliver the human immune stimulatory molecules HLA-A2, HLA-B13, and murine H-2K genes into a cutaneous metastasis resulted in

complete regression of two tumors, while four gave partial local response (Hui et al., 1997). Another trial using cationic liposomes to express HLA-B7 suggested that gene therapy using cationic liposome vectors for tumors would be both feasible and safe (Rubin et al., 1997).

Although cationic liposome technology has come a long way since the initial experiments, the levels of transgene expression conferred by the numerous cationic complexes is several orders of magnitude lower than efficient viral delivery systems such as Ad vectors. A disadvantage of liposomes is that once they are endocytosed by the target cell, they eventually become degraded. The use of the cationic polymer polyethylenemine, which contains numerous protonatable groups, improves properties of liposomes, as it is thought that the presence of protonable groups on the liposome may facilitate escape from the endosomes. Similar limitations apply to cationic liposome complexes as for pDNA, and, therefore, large amounts of DNA are required to transduce target cells in vivo. In spite of this, high levels of transgene expression are usually not generated in vivo. Studies using cationic liposomes express soluble factors, or requiring a low percentage of transfection, have the highest possibilities of being successful for in vivo applications.

An alternative is to use targeted *polyamine conjugates* that utilise the natural endocytosis pathway inherent to cells to gain efficient access into the cytoplasm. Targeted polyamine conjugates involve the production of polyamine-DNA aggregates, covalently linked to specific proteins on their exposed surfaces, which recognise specific epitopes on the surface of target cells. The polyamine condenses the DNA into tightly packed structures, which then can be coupled to any desired protein. The versatility of such a system is that any protein can be linked to the polyamine conjugate and could be targeted to any cell type within the body. Numerous receptors have been targeted using this system, including epidermal growth factor receptor and the ErbB2 receptor (Cristiano & Roth, 1996).

Viral/DNA hybrids have also been developed as gene transfer vectors. A well-characterized example of a viral hybrid is the Sendai virus (HVJ)/liposome complex. Here the F-protein from the HVJ is fused with liposomes containing DNA expressing the gene of interest. Due to its apparent efficiency, numerous transgenes have been expressed from the HVJ-liposome complex in vivo. Expression of HSV-1 thymidine kinase from HVJ-liposome complexes resulted in 80% of the meningeal gliomatosis mice to be cured when treated with ganciclovir, suggesting that this delivery system may be applicable for the in vivo gene therapy of human malignant glioma (Mabuchi et al., 1997).

Non-viral vectors can be modified by incorporation of fusion proteins (Saeki et al., 1997), targeting elements (Shi & Pardridge, 2000), high-mobility group proteins, nuclear localization signals (Aronsohn & Hughes, 1998), or viral elements (Kaneda et al., 1999) to improve, respectively, transfection efficiency, DNA transit into the nucleus, or DNA stability. However, despite these improvements, the transduction efficiency is still too low, considering gene delivery requirements to treat brain tumors.

4. Cancer gene therapy approaches

Many different anticancer gene therapy approaches are being developed, each with its own merits and limitations, but it is important to consider that it is unlikely that any of these strategies on its own will be a "magic bullet" for cancer cure. It is more probable that gene therapy will succeed when several strategies are used in combination, as in the classical pharmacological treatment of disease. Furthermore, as is the case for all of the ongoing human gene therapy clinical trials, it will be used in conjunction with traditional cancer treatments, such as surgery, chemotherapy, and radiotherapy.

This is not intended to be a detailed review of the many anticancer gene therapy strategies that exist and that are in varying stages of research and clinical development. However, it is important to highlight these approaches and to consider their potential merits; the ways in which they may be combined with conventional treatment strategies; and how, in particular, they may be exploited for the treatment of GB.

4.1. The correction of the primary genetic defect in cancer cells and/or to repair cell cycle defects caused by losses of tumor suppressor genes or inappropriate activation of oncogenes

Although the genetic alterations that give rise to a cancer cell may be numerous and differ between tumor types and individuals, there are frequently occurring gene mutations. Perhaps, the most common genetic alteration is in the p53 tumor suppressor gene, which is mutated in about one-half of the human tumors. Tumor suppressor genes encode for proteins that suppress cell division; therefore, when deleted or mutated, the control of cell growth is lost. By introducing an unmutated tumor suppressor gene into cancer cells, apoptosis can be induced and tumor growth inhibited.

Inactivation of p53 occurs early in glial tumorgenesis, and mutations are commonly found in low-grade astrocytomas (Frenkel & Wyatt, 1992). Replacement of a defective p53 gene has been described for many tumor models. Gomez-Manzano et al. (1996) used adenoviral delivery of a wild-type p53 gene into two glioblastoma cell lines expressing either a wild-type or mutant p53 gene. In these experiments, the wild-type p53 cells showed an inhibition in proliferation and mutant p53 cells underwent apoptotic cell death. In anaplastic astrocytomas, mutation of the retinoblastoma (Rb) gene and/or p16 gene is most common. The mutation, methylation, or deletion of p16 is also found in a variety of human tumor cell lines, including 80% of the glioma cell lines. Restoration of p16 expression, again using an adenovirus vector for delivery into p16-deficient glioma

cell lines, D-54M, U-251MG, and U-87MG produced growth arrest (Fueyo et al., 1998a). Similar experiments providing the Rb gene resulted in glioma cell survival in vitro (Fueyo et al., 1998b). Within glioblastoma, alterations to chromosome 10 have also been identified, indicating that a glioma-associated tumor-suppresser gene may reside within this region (Rasheed, 1992). Microcell-mediated transfer has been used to introduce chromosome 10 into tumorigenic U-251MG glioma cells, which when injected into nude mice, did not form tumors (Pershouse et al., 1993). Recently, a tumor suppressor gene, MMAC1/PTEN, was identified within this chromosome, which is commonly mutated in human glioblastoma (Rasheed et al., 1997). In vitro proliferation of U87MG cells was inhibited by MMAC1 expression and replication-defective adenovirus delivery of MMAC1/PTEN into U87MG cells rendered them almost completely non-tumorigenic, compared with untreated and controls (Cheney et al., 1998). This suggests that the in vivo gene transfer of MMAC1/PTEN could be potentially useful in cancer gene therapy for aggressive gliomas.

Alternatively, with increasing knowledge of the regulatory pathways of apoptosis-specific signaling molecules and novel cell surface-bound death receptors/ligands that regulate apoptosis, which are either deleted, mutated, or abhorrently expressed in cancer cells, replacement of these pro-apoptotic genes will also induce programmed cell death of tumor cells. Abnormalities in the apoptotic cascade are almost always present in tumor cells, including gliomas. Apoptosis can be mediated via the Fas/FasL (Fas ligand) pathway. An increase in cell surface expression of Fas in glioma cells increases their susceptibility to Fas antibody-mediated apoptosis in vitro (Weller, 1998; Weller et al., 1998). Our laboratory recently has generated recombinant adenovirus vectors expressing FasL under the control of cell type-specific promoters that exhibit reduced systemic toxicity (Larregina et al., 1998; Morelli et al., 1999). When used in an in vivo model of intracranial glioma, overexpression of FasL increased survival of tumor-bearing animals (Ambar et al., 1999).

4.2. Suppression of angiogenesis

Angiogenesis is the process of new blood vessel formation, which is critical for the growth of cancers above 1–2 mm. Consequently, tumor cells have developed mechanisms to induce angiogenesis and recruit new blood vessels. This intact tumor vasculature is an appealing target, as angiogenesis is down-regulated in healthy adults, so targeting should lead to minimal side effects, even after prolonged treatment. Tumor capillaries supply hundreds of tumor cells, indicating that targeting angiogenesis should lead to a potentiation of the antitumor effect, and direct contact of the vasculature with the circulation will allow efficient access of therapeutic agents (Rubin et al., 1997). Two main anti-angiogenic approaches currently in development are the inhibition of the angiogenic process and vessel formation by the delivery of anti-angiogenic agents or direct targeting and destruction of the tumor vasculature.

Studies of the angiogenic process have identified angiogenesis-related molecules as potential targets for cancer therapy. Amongst these targets are vascular endothelial growth factor (VEGF)/VEGF-receptor complex, α (αv) integrins, and Tie receptor kinases. Several experimental strategies have been investigated using VEGF, including the use of antisense and antibody approaches to inhibit VEGF and the use of soluble VEGF receptors. Stable C6 glioma cells expressing an antisense VEGF cDNA expressed reduced quantities of VEGF compared with parental cells, and these tumor cells showed reduced ability to form neoplasms, which correlated with a significant decrease in blood vessel formation (Saleh et al., 1996).

Resection of some human tumors, such as breast and colon carcinoma, can lead to the rapid growth of distant metastases, and it was proposed that the primary tumor had been suppressing growth of these metastatic lesions by the expression of anti-angiogenic factors. Angiostatin and endostatin are two such factors, which may hold potential for the treatment of disseminated cancers (O'Reilly, 1997; O'Reilly et al., 1997). Angiostatin, which is an internal fragment of plasminogen, has been purified from human plasma and the effects of its systemic administration on glioma tumor growth have been investigated. When C6, 9L glioma, and the human U87 glioma cell lines were implanted either subcutaneously or intracranially in nude mice, a growth inhibition was observed with angiostatin treatment, which was accompanied by significant reduction in vascularity. Most importantly, this tumoristatic activity against an intracranial tumor proved to be as potent, showing that it was independent of the blood-brain barrier (Kirsch et al., 1998). These encouraging results need to be taken with caution, since a recent report showed that endostatin was not able to control the progression of human B-lineage acute lymphoblastic leukemia in vivo in a mouse model of this disease (Eisterer et al., 2002). This therapeutic modality should be tested in stringent models of glioblastoma before it can become a possibility for human trials. Recently, a technique has been described for the treatment of malignant brain tumors based on local delivery of the anti-angiogenic protein endostatin from genetically engineered cells encapsulated in ultrapure sodium alginate (Read et al., 2001). The endostatin released from the capsules led to an induction of apoptosis, hypoxia, and large necrotic avascular areas within 77% of the treated tumors, whereas all the controls were negative. This delivery approach could be further developed to provide the basis for treatments for glioblastoma.

4.3. Activation of the immune response

Harnessing the strategies of the immune system is a very attractive approach to treat cancer. In this review, we will focus mainly on the development of immunotherapies to

treat astrocytomas, which are the most common type of brain tumors. Further, in their malignant form, the life expectancy of diagnosed patients is very poor, despite advances in surgical techniques, chemotherapy, and radiation therapy. This is due partly to the infiltrating nature of this tumor and its recurrences. To stimulate an efficient antitumor T-cell response in the CNS requires similar elements and events as for other organs or tissues, but due to the presence of the blood-brain barrier, which isolates the CNS from the rest of the body, and the scant lymphatic drainage of the CNS and the absence of any dendritic cells or analogous cells from the brain, stimulation of anti-brain tumor immunity is inhibited (Lowenstein, 2002). Areas of great importance are the elucidation of antigen presentation in the CNS, characterization of T-cells involved in tumor recognition, and the identification of specific tumor antigens.

It is now well established that tumor cells can interact with immune cells, and these interactions are either mediated by receptor ligand interactions, which allow cell-to-cell contact, or through a battery of cytokines. In the case of malignant astrocytomas, there is evidence that the host immune response is not capable of eliminating the tumor. Therefore, one therapeutic approach would be to use stimulatory cytokines, which could aid in generating an efficient antitumor immune response. A complementary strategy could be to block inhibitory cytokines such as TGF-β, and this could also contribute to generate an antitumor immune response. Another approach that has been actively pursued is to use either tumor antigens or genetically engineered tumor cells to immunize the host against the tumor (Table 3). T-cells generated by priming an antitumor immune response in the periphery, recirculate and reach the brain, where they can mediate antitumor cytolytic effects. In the following sections, we will discuss some of the immune-based strategies that could be implemented for the treatment of malignant astrocytoma or glioma.

Table 3
Immune therapeutic strategies for GB

Cytokine-mediated immunotherapy
• Interferons
• Interleukins
• TGF-β antagonists
Antibody-mediated immunotherapy
• Monoclonal/polyclonal
• Conjugates (isotopes, toxins, cytotoxic drugs)
Immune cell-mediated tumor killing
• Natural killer cells
• Lymphokine-activated killer cells
• Tumor-infiltrating lymphocytes
• Specific cytotoxic lymphocytes
Vaccination
• DC fusions
• DCs pulsed with tumor antigen(s)
• Autologous tumor-cell immunization
Harnessing death ligand/death receptor interactions
• FasL-mediated tumor cell death
• AP02L-mediated tumor cell death
• TNF-α
Gene-based immunotherapy
• Inhibition of immunosuppressive factors, i.e., soluble TGF-β receptors
• Gene transfer of interferons, interleukins, or TNF-α
• Gene transfer of death receptors or death ligands
• Activation of antigen-presenting cells

4.3.1. Enhancement of the immune response using cytokines

Chemokines are primarily involved in the recruitment of different types of leukocytes to tissues/organs, and can also modulate angiogensis and hematopoiesis. They have modulatory actions on natural killer (NK) cells and dendritic cells (DC), and these cells may provide tools to activate specific antitumor immune responses.

One of the main cytokines used for tumor treatment has been TNF-α. Although TNF-α can induce death of a few types of tumor cells, its main functions are to regulate a broad range of immune-mediated responses, i.e., stimulate proliferation of T-cells; enhance cytotoxicity of granulocytes and natural killer cells; induce the secretion of several other cytokines, such as IL-2, IL-6 platelet-derived growth factor (PDGF); and induce the expression of IL1-β and ICAM-1. It also induces up-regulation of MHC class I and II expression on immune cells (Romanczuk et al., 1999). TNF-α can induce expression of CD95 on glioma cells, and this could be harnessed in the development of death—receptor-mediated tumor killing (Schwartz et al., 1996). To date, there are no encouraging data of either preclinical or clinical outcomes after using TNF-α for treating brain glioma (Yoshida et al., 2001). TNF-α might be beneficial in the treatment of brain glioma when used in conjunction with other tumor-killing strategies, such as conditional suicide gene therapy using herpes simplex virus type 1 thymidine kinase (HSV1-TK) in the presence of ganciclovir (GCV) (Moriuchi et al., 1998). When radiosurgery was included as an additional treatment option, the combination of these three therapies, i.e., HSV1-TK plus GCV, TNF-α overexpression, and radiosurgery, yielded the best survival outcome (Niranjan et al., 2000). These results suggest that TNF-α, in combination with other treatment strategies, might provide additional benefit to glioma patients.

Another cytokine that has been proposed for treating gliomablastoma multiforme is IL-4. IL-4 is not cytotoxic to glioma cells in vitro, but, nevertheless, it has been proposed to treat glioma in situ, due to its capability of inducing a very strong immune response when administered in the brain parenchyma (Yu et al., 1993). IL-4 also has antiproliferative effects on human glioblastoma cells in vitro and when transplanted into athymic nude mice in vivo (Topp et al., 1995; Wei et al., 1995, 1998). IL-4 has been co-expressed with HSV1-TK, and after treatment with GCV, 38% of tumor-bearing Sprague-Dawley rats survived for at least 2 months (Benedetti et al., 1997). These results indicate that combination therapies are more effective when compared with single modality treatments. Virally mediated gene transfer of IL-4 in several preclincial animal models of brain glioblastoma has shown successful tumor regression

(Benedetti et al., 1999; Saleh et al., 1999). IL-4 has been expressed as a fusion protein to the translocation and enzymatic domains of pseudomonas exotoxin in several glioma animal models and in phase I clinical trials in human patients (Puri et al., 1996; Husain et al., 1998; Puri, 1999; Joshi et al., 2002).

IL-2 and IFN-γ have also been tested as potential adjuvants for the treatment of brain tumors. Co-implantation of RG-2 glioma cells and retroviral vector producer cell lines providing a local source of IL-2 or IFN-γ initiated a cell-mediated antitumor response in vivo (Tjuvajev et al., 1995). This resulted in early suppressing of intracranial tumor growth, but there was no increased survival of the animals, which was possibly due to severe CNS toxicity caused by IL-2 and IFN-γ production (Tjuvajev et al., 1995). Expression of IL-10 in combination with IL-2 also had significant immune-dependent antitumor actions within intracranial gliomas (Book et al., 1998). Regression of brain tumors established in naïve mice has also been demonstrated by combined administration of an intratumoral injection of a single dose of IL-2 producing glioma cells and recombinant IL-12. Mice cured of their intracerebral tumors displayed protective immunity upon rechallenge (Kikuchi et al., 1999). Previous studies have also shown that vaccinia virus expressing p53, IL-2, and IL-12 resulted in effective inhibition of subcutaneous glioma growth in mice; similar effects were obtained in a glioma nude mouse model (Chen et al., 2000). Intracerebral transplantation of IL-2 producing rat gliosarcoma 9L cells and subcutaneous vaccination using irradiated 9L cells could cure 9L-bearing tumor rats. These results suggest that a strategy combining IL-2 production within the tumor mass, together with immunization with irradiated whole tumor cells, could be a potential strategy for brain tumor treatment (Iwadate et al., 2001).

IFN-γ has significant antiproliferative effects on glioblastoma cell lines in culture (Kominsky et al., 1998). These results could also be seen in vivo after immunizations with transfected glioma cells expressing IFN-γ and IL-7, which resulted in significant growth inhibition of the intracerebral parental tumors (Visse et al., 1999). Intratumoral administrations of liposomes containing the murine IFN-β resulted in a 16-fold reduction in the mean volume of intracranial gliomas in the brains of C57BL/6 mice and also massive infiltration of cytotoxic T lymphocytes (CTLs) within the residual tumor, further, 40% of treated mice were completely cured (Natsume et al., 1999). IFN-β therefore, might, activate an effective antitumor immune response in vivo (Natsume et al., 2000) and also inhibition of tumor angiogenesis (Saleh et al., 2000). Although it has been reported that in a phase III study of radiation therapy plus BCNU with or without recombinant IFN-α for the treatment of patients with newly diagnosed high-grade glioma, there was no significant difference with regard to time of disease progression and overall survival between these two groups (i.e., plus/minus IF-α) (Buckner et al., 2001). More so, in this trial, IFN-α appeared to add significant toxicity. This could be due to the high doses of IFN needed to achieve beneficial pharmacological doses and also the short half-life of this molecule. In this scenario, gene therapy could prove invaluable by providing a continuous delivery of IFN-α at the tumor site and also bypassing the need for this molecule to go through the blood-brain barrier.

4.3.2. Enhancement of T-cell activation

Another way of increasing an immune response against a tumor is to enhance T-cell activities. T-cells recognize foreign peptides bound to MHC molecules, and for activation, they also require simultaneous delivery of a co-stimulatory signal by a specialized antigen-presenting cell. The most potent activators of naïve T-cells are mature dendritic cells, and these cells initiate T-cell responses in vivo. The best-characterized co-stimulatory molecules are glycoproteins B7.1 and B7.2, which are members of the immunoglobulin superfamily and are found exclusively on the surface of cells that can stimulate T-cell proliferation (Sharpe & Freeman, 2002). The receptor for B7 molecules on the T-cell is CD28. Once activated, T-cells express a number of molecules that sustain or alter co-stimulatory signals, i.e., CD40 ligand. CD40 ligand binds to CD40 present on antigen-presenting cells, stimulating further proliferation. Another pair of molecules that contributes to co-stimulation of T-cells are 4-1BB (CD1370) and its ligand 4-1BBL, expressed in activated dendritic cells, macrophages, and B-cells. Other CD20-related proteins are expressed on activated T-cells, i.e., CTLA-4 (CD152), which acts as a receptor for B7 molecules and delivers an inhibitory signal to the activated T-cell. This limits the proliferative response of activated T-cells to antigen and B7. This interplay has important implications for tumor immunotherapy by providing B7-CD28 co-stimulation, which can result in the activation of T-cells to lower affinity epitopes (Komata et al., 1997; Kwon et al., 1997; Greenfield et al., 1998; Liebowitz et al., 1998; Strome et al., 2000; Bai et al., 2001; Chambers et al., 2001).

Combination therapies using cytokines and co-stimulatory molecules have been performed in preclinical animal models with some degree of success. The human glioblastoma cell line D54MG was transduced in vitro with genes encoding the pro-inflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), the T-cell co-stimulatory molecule B7.2, or both (in a bisistronic vector) using retroviral vectors (Parney et al., 1997).

The effect of GM-CSF and/or B7.2 transduction on D54MG tumor growth in vivo was monitored in a novel allogeneic human peripheral blood lympocyte-severe combined immunodeficiency mouse (Hu-PBL-SCID) model. GM-CSF or B7.2 transduced tumors showed growth suppression in hu-PBL-reconstituted mice compared with untransduced and/or unreconstituted controls (Parney et al., 1997). Growth suppression was greatest for B7.2. Furthermore, vaccination with irradiated GM-CSF/B7.2 transduced tumor cells markedly inhibited growth of wild-

type tumors at distant sites. Thus, this study illustrates a potential gene therapy strategy for glioblastoma patients using GM-CSF and/or B7.2 transduced tumor vaccines (Parney et al., 1997).

The antitumor effects of this recombinant IL-12 and genetically engineered glioma cells expressing B7.1 or both B7.1 and ICAM-1 has also been examined (Joki et al., 1999). Vaccination of mice with B7.1-expressing tumor cells substantially inhibited the growth of subcutaneously inoculated gliomas, but not those located in the brain. Vaccination with B7.1-expressing tumor cells and systemic recombinant IL-12 (rIL-12) was more effective than either B7.1-expressing tumor cells or rIL-12 alone (Joki et al., 1999). Vaccination with tumor cells expressing both B7.1 and ICAM-1 combined with rIL-12 prolonged survival in a murine brain tumor model (Joki et al., 1999).

4.3.3. Manipulation of dendritic cells as a tool for brain cancer immunotherapy

A promising strategy for tumor treatment is to introduce cytokine genes into tumor cells so that they will secrete the appropriate cytokine in order to attract antigen-presenting cells to the tumor mass. Examples of these cytokines include GM-CSF and Flt3 ligand, which induce the differentiation of hematopoietic precursors into dendritic cells and attract them to the tumor site. These cytokines are also thought to act as adjuvants, activating the dendritic cells to process the tumor antigens and migrate to the local lymph nodes. Once within the lymph nodes, they would elicit potent antitumor responses (Avigan, 1999).

Another strategy for vaccinating against tumors is to use antigen-pulsed autologous dendritic cells (DCs) to stimulate antitumor cytotoxic T-cell responses. This approach has been used successfully in animal models (Aoki et al., 2001) and also in phase I/II human trials (Yu et al., 2001).

The challenges of these approaches are whether it will be possible to mount enough T-cell responses to either eliminate or control tumor growth, when most naïve tumor specific T-cells would be tolerant to the tumor, and whether an autoimmune response could be generated against normal tissue surrounding the tumor. Experimental data in preclinical animal models suggest that the continuous localized delivery of subcutaneous GM-CSF in conjunction with inactivated tumor antigens can initiate a systemic response that leads to the regression of distant peripheral intracerebral glioma tumors (Wallenfriedman et al., 1999). Tumor peptide-pulsed dendritic cell therapy led to prolonged survival in rats with established intracranial 9L tumors implanted 7 days prior to the initiation of vaccine therapy in vivo (Liau et al., 1999). Systemic vaccination of VM/KD mice with DCs pulsed with tumor homogenate followed by intracranial tumor challenge produced an increase in median survival compared with mice vaccinated with PBC or unpulsed DCs. Fifty percent of mice treated with pulsed DCs survived long-term (Heimberger et al., 2000). Immunologic memory was demonstrated in this study due to the survival of mice challenged with tumor. Both cell-mediated and humoral immunity was also induced (Heimberger et al., 2000).

In a phase I study, the feasibility, safety, and biosafety of an autologous peptide-pulsed dendritic cell vaccine for patients with malignant glioma was demonstrated (Yu et al., 2001). A pilot study of vaccinations with irradiated autologous glioma and dendritic cells, together with IL-4 transduced fibroblasts, to elicit an immune response, has also been performed in patients with malignant glioma (Okada et al., 2001). The safety and clinical response after immunotherapy with fusions of dendritic cells and glioma cells for the treatment of patients with malignant glioma has been assessed (Kikuchi et al., 2001). Although the results of the phase I clinicial trial of fusion cells indicated that this treatment safely induced immune responses, the authors were not able to establish a statistically significant treatment-associated response rate due to the limited sample population (Kikuchi et al., 2001). These results suggest that although DC therapy seems to be safe and induces an immune response, their efficacy to treat patients with malignant glioma needs to be further studied.

4.4. Harnessing death receptor-ligand interactions

One of the major challenges in glioma chemotherapy is that tumor cells usually acquire resistance to chemotherapeutic agents. Death receptors and their ligands recently have been proposed as alternative strategies for the treatment of malignant brain tumors, as direct activation of caspases would lead to tumor cell death. Members of this protein family include TNF-α; CD 95 ligand, and Apo2 ligand (AP02-L). Advantages of this approach include the fact that death receptor/ligand-induced cell death is independent of losses or mutations of p53, retinoblastoma, or p16 and also, of the immunosuppressive effects exerted by TGF-β, which is overexpressed by glioma cells and has been demonstrated to antagonize many immune therapeutic approaches (Roth & Weller, 1999). As with any other therapeutic options, there are disadvantages to this approach, i.e., low level or no expression of the appropriate death receptors on the surface of tumor cells. Also, under some physiological circumstances, death receptors might become more abundant in non-tumoral brain tissue and, therefore, death ligand-mediated therapies may extend overt toxicity. It has been reported that astrocytes of human origin can be susceptible to AP02-L-induced death in culture (Walczak et al., 1999). The same group did not show any toxicity in the brain in vivo in rodents. In F98 glioma-bearing rats, infection with a recombinant adenovirus vector expressing Fas-L increased the mean survival of the animals by 80% compared with infection with a recombinant adenovirus expressing the marker gene β-galactosidase or untreated controls (Ambar et al., 1999). In vitro, malignant glioma cells are sensitive to Fas-ligand-mediated cell death (Frei et al., 1998). We recently have also shown that human malignant glioma cells in primary culture that are resistant to CCNU become sensitive to Fas ligand-

mediated cell death (Maleniak et al., 2001). Human glioma cell lines are also susceptible to AP02-L-induced apoptosis (Rieger et al., 1998; Rohn et al., 2001). It recently has been reported that transfer of the gene encoding second mitochondria-derived activator of caspase (SMAC) sensitized various tumor cells in vitro and malignant glioma cells in vivo for apoptosis induced by death-receptor ligation or cytotoxic drugs (Fulda et al., 2002). SMAC peptides enhanced the antitumor activity of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in an intracranial malignant glioma xenograft model in vivo (Fulda et al., 2002). Taken together, these results show that death receptor/ligand-mediated cell death may become an attractive therapeutic modality for the treatment of brain glioblastoma, which could be used in combination with other treatment modalities. Before this can be implemented, there is a need to elucidate potential toxic side effects in the normal brain and also to increase the efficacy and diminish the putative side effects of gene transfer into the CNS.

4.5. Providing drug resistance to hematopoietic cells

Many anticancer drugs not only kill tumor cells, but also adversely affect hematopoietic cells such as the bone marrow, resulting in myelosuppression. Therefore, reducing this chemotherapy-induced toxicity to bone marrow might allow dose-intensification of existing chemotherapy drugs. One way to protect these hematopoietic tissues is by increasing the expression of genes that increase drug resistance.

Expression of the drug efflux pump P-glycoprotein, encoded by the multidrug resistance (MDR1) gene, has been identified as an impediment to successful eradication of tumors by chemotherapy. The expression of the MDR1 and another MDR protein (MRP) has been assessed in glioma patients before and after chemotherapy. In this study, the proportion of MRP-positive and MDR1-positive glioma cells increased after chemotherapy, suggesting that both of these genes may be involved in acquired drug resistance in human glioma.

However, these chemoresistance genes can be used to protect healthy bone marrow from the toxic effects of chemotherapy by transducing the bone marrow cells with, for example, MDR1. Resistance of tumor cells to alkylating anticancer agents has also been shown to correlate with the expression of O^6-alkylguanine-DNA alkyltransferase (ATase) (Baer et al., 1993). Alkylating agents specifically cross link DNA at the O^6 position of the nucleotide, guanine, which can be corrected by ATase. Therefore, alkylating agents have been co-administered with O^6-benzylguanine and related pseudosubstrates that are able to inactivate human ATase. However, ATase depletion can also have major deleterious effects on the hematopoietic system. Therefore, gene therapy of ATase into bone marrow is also being investigated as another mechanism of protection.

Most work in this area has focused on the use of recombinant retroviruses as vectors for the delivery of the MDR1 gene and/or O^6-methylguanine DNA methyltransferase into hematopoietic cells (Jelinek et al., 1999; Fairbairn et al., 2000). Retroviral-mediated transfer of the human MDR1 gene is also been through a phase 1 clinical trial in patients undergoing bone marrow transplantation as part of a high-dose chemotherapy treatment for advanced cancer (Hesdorffer et al., 1998).

4.6. Enzyme/prodrug gene therapy

As the toxicity of cytotoxic anticancer agents to normal cells is one of the major limitations in cancer chemotherapy, drug targeting to antigen or receptor sites that are overexpressed in tumor cells has been investigated to increase treatment specificity. This can be achieved by conjugation of the cytotoxic drug to an antibody or other molecule, which binds to the tumor-specific antigens/receptors. However, limitations of this approach, including heterogeneity of tumor antigens, low accumulation of the cytotoxic drug at the tumor site, and poor penetration throughout the tumor mass, have hampered these attempts.

An alternative to this has been the use of prodrugs, which are chemicals that are inert over a wide range of prodrug doses, but can be converted to pharmacologically active, toxic molecules by specific activating enzymes. The active drug can be of low-molecular weight so that it can quickly diffuse throughout the tumor mass, reaching regions that would be nonaccessible by antibody-conjugated cytotoxins.

Initially, endogenous mammalian enzymes that were expressed at higher levels in tumors, i.e., β-glucuronidase, were exploited. However, this natural susceptibility proved to be rare amongst many malignancies. This approach was limited still further as most normal cells also expressed these enzymes, albeit at a lower level. So, to expand the range of tumor types susceptible to suicide therapy and to reduce the systemic toxicity of the prodrugs, many non-mammalian prodrug-activating enzymes have been identified.

4.7. Antibody-directed enzyme prodrug therapy

To produce the widest therapeutic potential between tumor and normal cells, prodrug-activating enzymes have been targeted to tumor cells using antibodies associated to tumor antigens in an approach termed ADEPT (Antibody Directed Enzyme Prodrug Therapy). The clinical strategy for ADEPT is to administer the enzyme-antibody conjugate to a patient and, after a time delay, to allow tumor localisation and natural clearance of any unbound enzyme-antibody conjugate from the blood, the prodrug can be administered at a nontoxic dose. This prodrug is then activated by tumor-localized enzyme to elicit production of toxic drug specifically at the tumor site. In addition, the time delay for the removal of any unbound enzyme-antibody conjugates prior to prodrug administration can be accelerated using a second 'clearance' antibody directed towards the enzyme (Rogers et al., 1995).

Aside from the specific tumor targeting of ADEPT, its key advantage lies in the potential ability of a single enzyme molecule to generate many hundreds of active drug molecules from the prodrug. This amplification effect contributes to what is termed the "bystander effect." Following conversion of the inert prodrug, the cytotoxic drug can be transferred to untargeted cells through gap junctions or can diffuse through the tumor mass to reach and kill tumor cells. This transfer of active metabolite also contributes to the bystander effect. It is also thought that as the active drug exerts its effect on transduced cells and apoptosis is induced, this will release apoptotic vesicles that may be phagocytosed by the untransduced cells. Once inside the untransduced cell, these vesicles are thought to be toxic, thereby inducing apoptosis in these cells. The final component of the bystander effect exerted by ADEPT can only be measured in vivo, where the immune response against the tumor-targeted antibody/enzyme conjugate also plays a role.

Limitations of using ADEPT arise from the low number of tumor-specific antigens that have been identified so far, restricting the use of ADEPT approaches to the treatment of mainly colorectal, breast, and choriocarcinoma tumors. This approach has also achieved insufficient enzyme delivery to the tumor, because the level of tumor-bound conjugate is dependent upon the level of expression of the target antigen. Furthermore, not all of the tumor may be accessible to the conjugate, restricting its free diffusion throughout the tumor mass. From initial studies using an antibody-enzyme conjugate constructed with a bacterial enzyme and a murine monoclonal antibody, the potential problem of immunogenicity of proteins of non-human origin has also been highlighted. This problem can be overcome by the use of immunosuppressive agents, and newer ADEPT systems are using human antibody-human enzyme fusions. In addition, the purification of antibodies is both time-consuming and costly, and issues of stability of the antibody-enzyme conjugate must also be considered.

Despite these limitations, ADEPT has progressed into phase 1 clinical trials using the bacterial prodrug-activating enzyme carboxypeptidase G2 (CPG2) conjugated to the F(ab′)2 fragment of a monoclonal antibody against carcinoembryonic antigen (CEA), followed by administration of the prodrug CMDA to treat patients with metastatic or locally recurring, non-resectable colon carcinoma (Martin et al., 1997; Napier et al., 2000). This approach could be adapted for the treatment of GMB, although due to the presence of the blood-brain barrier, the conjugates would need to be administered intratumorally or within the tumor bed at the time of surgical resection.

4.8. Gene-directed enzyme prodrug therapy and virus-directed enzyme prodrug therapy

As an extension of ADEPT and to overcome some of its limitations, the idea of gene-directed enzyme prodrug therapy (GDEPT) was developed. GDEPT uses vectors to introduce a gene encoding for a prodrug-activating enzyme into tumor cells. It is designed to be a two-step therapy in which the gene is delivered into the tumor tissue, and as it is encoded within an expression cassette, is transcribed to produce active enzyme. The non-toxic prodrug can then be administered systemically, and upon reaching the transduced cells, it is converted to its cytotoxic metabolite. The vector will target and transduce the tumor and the cytotoxic drug will be generated directly at the tumor site. In theory, this approach will give an enhanced amplification effect over ADEPT, as many enzyme molecules can be synthesized from a single expression cassette. In turn, each of these individual enzyme molecules can activate many hundreds of prodrug molecules.

Using GDEPT, the subcellular localization of the enzyme can also be controlled. The enzyme can be expressed intracellularly as an extracellular protein bound to the plasma membrane or secreted from the cell. It could also be secreted as a fusion protein, for example, fused to a carrier protein such as VP22 (Elliott & O'Hare, 1997). The herpes simplex virus type 1 tegument protein VP22 has been shown to mediate intercellular transport of any protein that has been fused to it. In this way, limitations of the gene delivery vehicle can be reduced, as the VP22 protein is able to carry the enzyme to surrounding untransduced cells.

Gene delivery of prodrug-activating enzyme can be achieved ex vivo, which allows for selection of transfected cells prior to their re-introduction into the patient. However, the number and types of cells that can be modified in this manner is limited, and even for accessible cells such as bone marrow precursor cells, the procedure is technically demanding. In addition, in most cases, ex vivo transduction will need to be patient-specific, becoming even more labor intensive.

The majority of GDEPT approaches use viral-mediated vector delivery, exploiting the innate ability of viruses to enter and mediate expression of transgenes in infected host cells. The use of viruses to deliver prodrug-activating enzymes is termed Virus Directed Enzyme Prodrug Therapy (VDEPT).

4.9. Enzyme/prodrug combinations

The list of enzyme/prodrug combinations that are being developed for cancer gene therapy is large, with each having its own merits and drawbacks. However, the two most advanced pairings are herpes simplex virus type 1 thymidine kinase/ganciclovir and cytosine deamine/5-fluorocytosine, both of which are in numerous gene therapy clinical trials. The bacterial enzyme carboxypeptidase G2, in combination with the prodrug CMDA, is the only enzyme to have reached clinical trial stage using ADEPT. Other, enzyme prodrug systems include *Escherichia coli* nitroreductase/CB1954 (Bridgewater et al., 1995, 1997), cytochrome P450 2B1/cyclophosphamide (Jounaidi et al., 1998), and

purine nucleoside prodrugs activated by viral thymidine phosphorylase (Hughes et al., 1998).

4.10. Herpes simplex virus type 1 thymidine kinase/ganciclovir

Thymidine kinase isolated from HSV1 is the most intensely studied prodrug-activating enzyme and has been used extensively in both preclinical and clinical studies to treat the majority of different solid tumors (Ram et al., 1997). First developed as a prodrug-activating enzyme by Moolten (1986), its substrates include the anti-herpes drug ganciclovir, which it phosphorylates to an intermediate. This intermediate is subsequently phosphorylated by cellular kinases to a di- or triphosphate form that can be incorporated into DNA as a nucleoside analogue. Phosphorylated ganciclovir can only kill dividing cells. Therefore, as tumor cells are usually actively dividing, it can be argued that this gives the therapy a level of selectivity similar to S-phase chemotherapeutic drugs. However, resistance to ganciclovir has been observed, which has been linked to the number of cells in resting G_0 at the time of GCV administration (Golumbek et al., 1992). Also, this approach prevents DNA repair.

The HSV1-TK/GCV system exhibits a strong bystander effect in vitro and in vivo. In fact, the term "bystander effect" was first coined for the HSV1-TK/GCV pairing when it was observed that transduced cells expressing HSV1-TK conferred chemosensitivity to neighboring untransduced cells (Freeman et al., 1993). Further proof for the existence of a bystander effect came from murine glioma tumor studies showing that only 10% of tumor cells needed to be transduced to achieve total tumor regression (Chen et al., 1995). To achieve a bystander effect in vitro using HSV1-TK/GCV requires cell-to-cell contact to allow the transfer of the GCV-triphosphate to untransduced cells. The requirement of cell-to-cell contact has been attributed to the fact that GCV-triphosphate is transmitted through gap junctions. In cell lines that lack gap junctions, for example, neuroblastoma and rat glioma cells, there is no bystander effect unless the cells are engineered with connexin genes to augment gap junction expression (Dilber et al., 1999).

The contribution that the host immune response makes to the bystander effect in vivo was also studied following HSV1-TK/GCV treatment. It was hypothesized that a host immune response was being generated against a non-human enzyme and also that the death of transduced tumor cells may stimulate immune recognition of tumor antigens and lead to the subsequent immune-mediated death of non-transduced tumors (Barba et al., 1994; Gagandeep et al., 1996). Using HSV1-TK/GCV, the immune-mediated bystander effect could eliminate the whole tumor when only a fraction of its tumor cells expressed HSV1-TK, and also, in immune-competent animals, it could result in the immune-mediated regression of anatomically distant tumor metastases (Kianmanesh et al., 1997).

Vector delivery systems for HSV1-TK have included cationic liposomes (Yoshida et al., 2001), herpes simplex virus (HSV), adenovirus, and retrovirus vectors. When HSV1-TK is delivered using replicating HSV vectors, GCV itself inhibits the propagation of the vector. This has allowed replicative virus to be used, with the aim of increasing the percentage of tumor cells, which can be transduced, using GCV as a useful safety mechanism to prevent uncontrollable viral spread.

The mutant HSV vector hrR3 is capable of replication, which itself is cytotoxic to cells and also encodes for an intact thymidine kinase gene so that the addition of ganciclovir to hrR3-infected cells may enhance the ability of hrR3 to destroy tumor cells. To increase specificity for tumor cells, hrR3 has a mutated ribonucleotide reductase gene and replicates selectively in cells with high levels of endogenous ribonucleotide reductase. Actively dividing cells such as tumor cells have high levels of endogenous ribonucleotide reductase for synthesis of DNA precursors. HrR3 has been proposed as a particularly good vector for the treatment of brain tumors because it can replicate in dividing gliosarcoma tumor cells, causing their lysis, but cannot replicate in postmitotic neural cells (Boviatsis et al., 1994; Mineta et al., 1994; Spear et al., 2000).

The ability to deliver viral vectors to intracranial tumors by injection into the carotid artery rather than by intracranial injection into the tumor has been investigated using hrR3 in an intracranial 9L gliosarcomas rat model. RMP-7, a bradykinin analog, was used to selectively open the blood-tumor barrier immediately prior to virus application. Viral delivery was essentially specific to tumor cells, with little transgene expression elsewhere in the brain, and there was minimal uptake by the kidney, spleen, and liver (Barnett et al., 1999).

On the basis of the good preclinical data for HSV1-TK/GCV and because GCV, as an anti-herpetic agent, has a good toxicity profile, HSV1-TK/GCV has been used in numerous clinical trials for many different malignancies. Two phase I–II clinical trials of HSV1-TK gene therapy, used immediately following surgical resection in patients with recurrent GB, have been carried out (Klatzmann et al., 1998; Shand et al., 1999). Retrovirally transduced fibroblasts expressing TK were administered by intracerebral injection immediately after tumor resection. From these trials, even though some regression was observed in some patients treated by gene therapy, the overall survival of patients after HSV1-TK gene transfer and GCV was similar to the results reported from resection plus chemotherapy (median survival 29–37 weeks, respectively). However, the gene therapy trials were all phase I clinical trials. We will need to await the results of the larger phase II and phase III trials.

One of the problems highlighted by the clinical trials of HSV1-TK/GCV is that gene transfer has been confirmed as very low; therefore, insufficient numbers of tumor cells may have been transduced. Attempts to increase the level of tumor transduction, aside from the use of replicative vectors

such as hrR3, include the fusion of TK to VP22 protein, which has been shown to facilitate intercellular spread of the HSV1-TK enzyme to non-transduced cells (Dilber et al., 1999). However, the dangers of spread to normal tissue must be recognized in this approach. Other attempts to increase the efficacy of HSV1-TK-mediated tumor killing have been the development of optimal prodrugs for TK, and the enzyme itself has been mutated to generate an enzyme with increased catalytic activity (Qiao et al., 2000). To limit the toxic effects of HSV1-TK/GCV on normal tissue, HSV1-TK expression can be controlled by using cell type specific promoters. A recombinant adenoviral vector has been constructed in which the HSV-TK gene is driven by the promoter for the gene-encoding glial fibrillary acidic protein, an intermediate filament protein expressed primarily in astrocytes, which has been used to shown to specifically kill GFAP-positive glioma cells (Vandier et al., 2000).

Work from our laboratory has shown efficient tumor eradication in a syngeneic rat tumor model that uses Lewis rats with intracranially implanted CNS-1 cells (Dewey et al., 1999) (Fig. 3). The long-term survivors after successful gene therapy developed side effects, which included cytotoxic demyelination, chronic active brain inflammation, and lymphocyte infiltration within the normal brain tissue (Dewey et al., 1999; Cowsill et al., 2000). Another striking observation of this study was the persistence and widespread distribution of the transgene HSV1-TK throughout the brain (Dewey et al., 1999; Zermansky et al., 2001). These findings have critical implications for the implementation of clinical trials for GB, which are summarized in Fig. 4. Of importance is the long-term persistence of the transgene expression in the brain, which suggests that one should be able to re-treat the patients with GCV without the need of repeated vector administration into the CNS (Fig. 4).

A powerful experimental approach would be the combination of the conditional cytotoxic tumor killing, (i.e., HSV1-TK in combination with GCV) with an immunestimulatory approach by using Flt3-ligand, which would induce the migration and differentiation of dendritic cells to the tumor site, facilitating the generation of a potent antitumor response in vivo (Fig. 5).

4.11. Cytosine deaminase/5-fluorocytosine

Cytosine deaminase is ubiquitous within bacteria and fungi, but absent from mammalian cells, and is useful for suicide gene therapy because it deaminates the antifungal drug 5-fluorocytidine into 5-fluorouracil. 5-Fluorouracil induces cell death by inhibiting both DNA and RNA synthesis, therefore CD/5-FC treatment can only kill dividing cells. Loss of cell contact via gap junctions is a characteristic of many cancers. Therefore, one of the major advantages of this system is its very strong bystander effect, which, unlike that of HSV1-TK/GCV, does not require cell-to-cell contact, but rather 5-FU can diffuse to untransduced cells. Experiments on subcutaneous tumors suggest that as few as 2–4% of the tumor cells need to be transduced to express CD to achieve significant regression or elimination of the tumor (Huber et al., 1993, 1994). However, this diffusion is not restricted to the tumor mass and may result

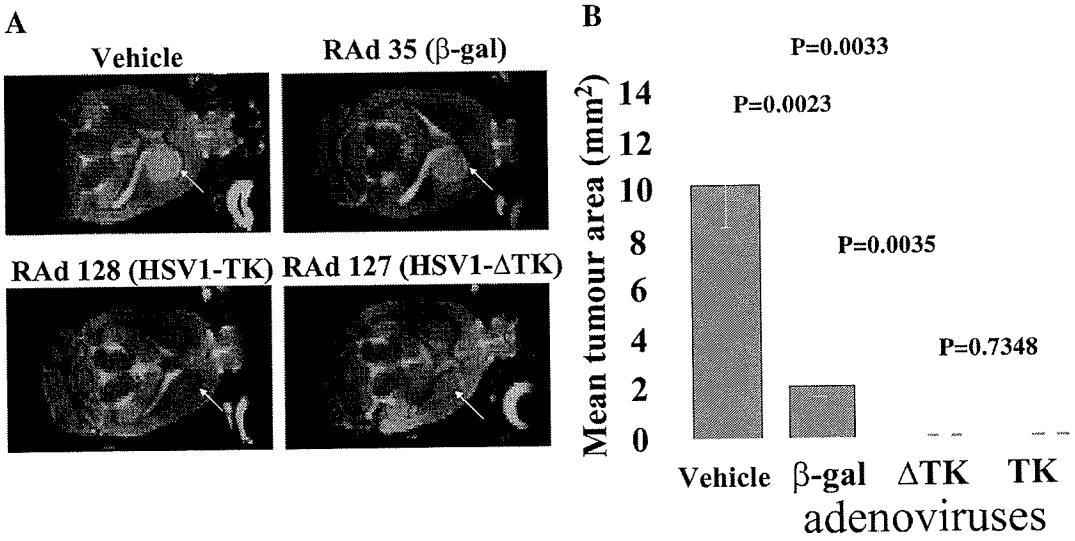

Fig. 3. Successful preclinical gene therapy trial in a syngeneic rat glioblastome model. Lewis rats were implanted with CNS-1 glioma cells in the striatum, as described in Dewey et al. (1999). Three days after tumor implantation, rats were subdivided into four groups and tested with either vehicle: rAd 35, a control recombinant adenovirus vector expressing the marker gene β-galactosidose, and rAd 128 on rAd 127, recombinant adenovirus vectors expressing the therapeutic genes HSV1-TK on a truncated form of it, i.e., HSV1-ΔTK. **A**: Shows in-vivo monitoring of tumor progression using proton magnetic resonance imaging of the rat brain. **B**: Shows the effects of recombinant adenoviruses on the growth of the tumors. Note the complete absence of a detectable tumor mass in the groups of animals treated with either rAd-HSV1-TK or rAd-HSV1-ΔTK.

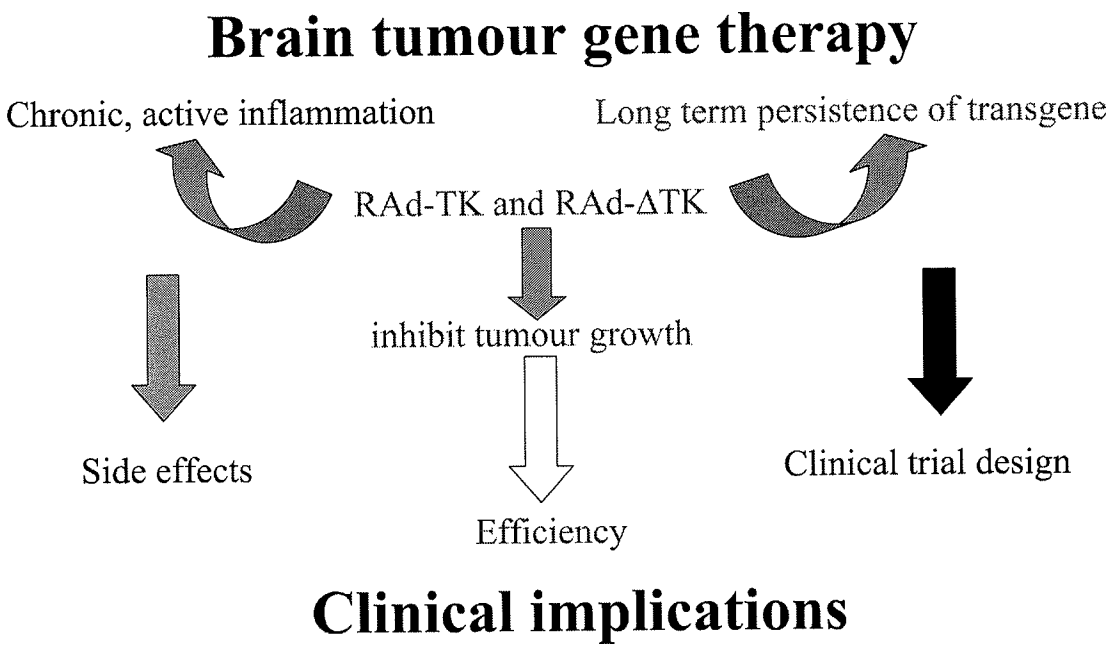

Fig. 4. Implications of successful preclinical brain tumor gene therapy using recombinant adenovirus vectors expressing HSV1-TK for the implementation of human trials. Effective inhibition of tumor growth and long-term survival of tumor-bearing animals was achieved after treating them with a recombinant adenovirus expressing HSV1-TK. Side effects to the treatment included chronic brain inflammation and dimyelination, which will need to be taken into account when designing a clinical trial. Also, the long-term persistence of therapeutic transgene expression has beneficial implication for clinical trial design, as the vector would not need to be administered repeatedly into the CNS.

in damage to normal tissue. CD has been delivered using the majority of viral and non-viral gene transfer vectors. Stereotactic injection with an adenovirus encoding for CD into an intracranial rat glioma model showed increased survival times, but large areas of brain necrosis resulting in death (Dong et al., 1996). Adenoviral vector delivery of CD is currently in human clinical trials to treat metastatic colon cancer and also metastatic breast cancer, in which the CD gene is under the control of the erbB2 promoter (Pandha et al., 1999).

4.12. Carboxypeptidase G2

CPG enzymes were first envisaged as direct chemotherapeutic agents in their own right because of their ability to deplete folic acid by hydrolytic removal of its glutamate moiety. Reduced folates play a key role as coenzymes in many biochemical pathways, but specifically in DNA synthesis, via their involvement in purine and pyrimidine biosynthesis. Tumor cells characteristically have a high rate of nucleic acid synthesis to support their rapid growth rate. Therefore, folic acid depletion can inhibit their growth, or even have a cytotoxic effect upon tumors with a high folic acid requirement (Rosenberg & Mason, 1989). This strategy of folate depletion is a standard and important technique in cancer therapy, as well as for dermatologic disorders (psoriasis) and rheumatologic disorders (severe rheumatoid arthritis).

The CPG enzymes also hydrolyse subfragments of folic acid, in particular glutamated benzoyl nitrogen mustards. These are bifunctional alkylating agents, but are deactivated because the effect of an ionised carboxyl group on the reactivity of the mustard is reduced by the linkage of the benzoic acid moiety through an amide bond to a glutamic acid residue. Cleavage of this residue by CPG2 releases a cytotoxic class of molecules, the nitrogen mustards. CPG2 does not have a human homologue, ruling out the possibility that nitrogen mustard prodrugs will be activated by a host enzyme at a non-tumor site. Unlike many other prodrug-activating enzymes that convert their prodrugs to an intermediate metabolite, which requires further catalysis by cellular enzymes, CPG2 alone can activate nitrogen mustard prodrugs. This eliminates the requirement for an endogenous cellular enzyme within tumor cells, which, in turn, can lead to drug resistance and the preferential survival of tumor cells with a defect or deficiency in the required cellular enzyme. In addition, mustard-alkylating agents are not cell-cycle specific and, therefore, can kill proliferating and quiescent cells.

Significant antitumor effects using antibody-CPG2 conjugates have been achieved in a number of mouse human tumor xenograft models. Immunospecific antitumor effects have been demonstrated with administration of CPG2 chemically linked to the $F(ab')_2$ fragment W14, specific for the β-subunit of human chorionic gonadotrophin (Springer et al., 1991) and also to A5B7 directing binding

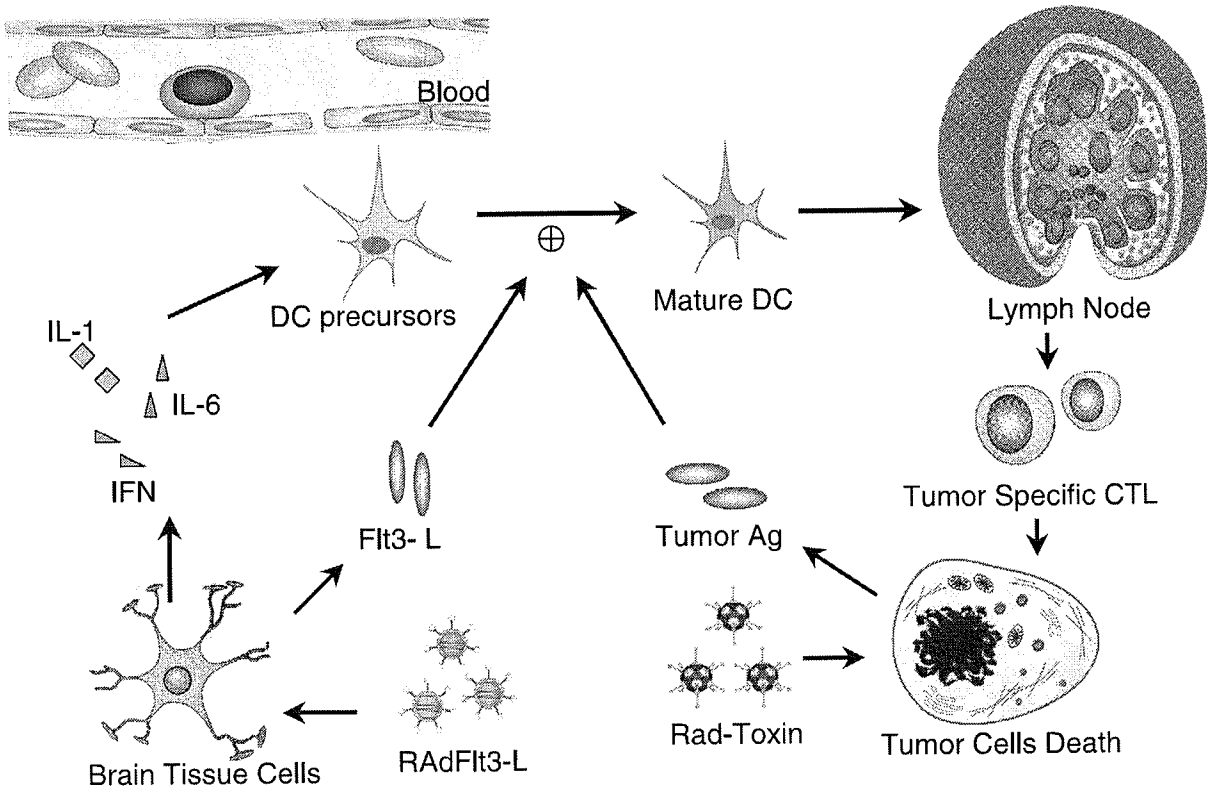

Fig. 5. Diagram illustrating immunotherapeutic approaches that could be implemented using gene therapy to treat brain glioblastoma. A recombinant adenovirus vector encoding the hematopoietic growth factor, Flt3-Ligand (Flt3-L), is used to inhibit DCs into the brain parenchyma within peritumoral regions. This could be done in conjunction with a conditional cytotoxic approach, i.e., HSV1-TK, which after peripheral delivery of ganciclovir, should cause the death of proliferating tumor cells, with concomitant release of tumor antigens. DCs should then take up these tumor antigens and mobilize them to the local lymph nodes, resulting in a specific antitumor immune response mediated by cytotoxic T-lymphocytes.

to the carcinoembryonic antigen (CEA) antigen for targeting to colorectal carcinomas (Blakey et al., 1995). CEA is also expressed in the majority of non-small cell lung, pancreatic, and stomach cancers and some small cell lung cancers. Targeting to ovarian (Springer et al., 1991) and breast carcinoma xenografts (Eccles et al., 1994) has also been achieved.

5. Validation of cancer gene therapy strategies in vitro and in vivo

In order to validate any new therapeutic strategies before taking them to clinical trials, there is a critical need to test their efficacy and safety using both in vitro and in vivo models. Below we will review the most commonly used systems for such validations.

Using primary cultures of cells derived directly from the resected tumors would provide cultures that are the most representative of the primary tumor, since they have not gone through any selection process. Alternatively, short-term cultures that have been passaged in order to increase cell numbers can also be used. Gene therapy strategies for the treatment of glioma can be tested using short-term cultures, as $\sim$90% of surgical biopsies of human glioblastomas can be established as short-term cultures. Yet, there are disadvantages to using these short-term cultures, which can sometimes be unstable, slow growing, and have a limited life span (Haselsberger et al., 1996).

Using primary cell cultures established from resected human brain astrocytomas, we have shown that gene therapy approaches are effective at tumor killing, even in situations were the cells were resistant to classical chemotherapeutic agents (Maleniak et al., 2001). These results suggest that in a clinical setting, the combination of classical treatment strategies with gene therapy might prove effective at controlling tumor burden.

Tumor cell lines that have been established for long-term culture are the most common choice for in vitro studies, and there are numerous glioma cell lines from both human and rodent species from which to choose. However, the growth rate and morphology of these cell lines should be properly documented to allow comparisons to be made with results generated by other research groups. Continual passaging has been shown to introduce heterogeneity into these cell lines and altered DNA profiles (Bradford et al., 1997).

To provide the fundamental toxicity and statistical survival data required prior to human clinical trials, treatment

strategies showing efficacy in vitro must be tested in in vivo animal models. Animal modeling of human tumors has proved invaluable for evaluating the efficacy of chemotherapeutics, targeted radiotherapy, and, most recently, gene therapy, including the determination of the potential benefits afforded by new anticancer therapies against brain tumors.

Rats and mice are most commonly used for modeling human tumors because they have well understood physiology and genetics and are easy to handle and maintain. Spontaneous tumors occur in the majority of non-human mammals at roughly the same incidence as they do in humans, but they are extremely rare in laboratory rodents. However, rodents develop tumors very quickly following exposure to chemical carcinogens or as a result of infection with an oncogenic virus. These induced tumors can be removed and maintained by subcutaneous passage or cell culture in vitro. These murine tumor cell lines or tissue explants can then be easily transplanted into other rodents of the same strain, which are termed syngeneic for the tumor.

Alternatively, human tumor cell lines or murine cell lines can be implanted into immunodeficient rodents to form tumors. This heterologous transplantation of tissue or derived cell lines was facilitated by the generation of the nude mouse, although previous implantation of human tissue into immune privileged sites, such as the eye and brain, had achieved some limited success. The autosomal recessive nude gene in homozygous (*nu/nu*) mice causes the lack of fur and an abnormal thymus, so these nude mice must be maintained in gnotobiotic isolators. The deficiency in T-cell function allows athymic mice to accept and grow xenografts, as well as allografts of normal and malignant tissues, without the mounting of an adaptive immune response. Heterozygous (*nu/+*) animals carry the recessive nude gene on one chromosome only and, therefore, have a normal thymus-triggered immune system. Characterization of human tumor xenografts grown in these athymic mice has demonstrated that the tumors retain many of the morphological characteristics of the original biopsy.

A further alternative is to use transgenic mice. Transgenic models have facilitated studies into the effects of tumor-associated oncogene expression, aiming to expose the complex process of oncogenesis. However, transgenic model generation is still in its infancy and is not a trivial undertaking. Even in the most well-planned experiments, the success rate is poor, with random integration into silent genomic areas or resulting in unexpected tissue expression. In addition, cancer arises when a single cell begins proliferating within normal tissue, unconstrained by the controls imposed by normal cell-to-cell contact. In transgenic mice, the tumor cell is surrounded by cells all containing the mutated gene, which may affect the tumor biology of these tumors.

As with all in vitro studies, animal models have their shortcomings, which must be acknowledged. None of the currently available animal models exactly simulates human tumors, each having strengths and weaknesses. It is essential to recognize that these will be dependent upon the nature of the study in which the model is to be exploited. Animals and humans have important metabolic, physiological, and hormonal differences, which will affect, for example, the way they metabolize carcinogens, therefore, having a major influence upon the testing of new therapeutic approaches.

5.1. Rat glioma models

Since the implantation of the carcinogen methylcholanthrene into the brains of mice, it has been known that primary CNS tumors could be chemically induced. However, only with the development of the synthetic carcinogenic drug class N-nitrosoureas (NNU) in the 1960s could brain tumors be induced selectively and with reproducibility. Direct brain application of N-ethylnitrosurea (ENU) or N-methylnitrosourea (NNMU) on laboratory rodents induced CNS tumors (Druckrey et al., 1968; Schmidek et al., 1971), and if administered to pregnant rats, transplacental oncogenicity induced tumors in all of the offspring (Koestner et al., 1971).

Concurrently, experimenters observed that non-tumorigenic virus, when innoculated at high titer into a non-permissive host, could also induce CNS tumors. The RNA virus, Rous sarcoma virus (RSV)-1 has most commonly been used to induce intracerebral sarcoma or glioma, firstly in chickens, then in dogs (Bigner et al., 1969). DNA viruses, such as Adenovirus type 12 and Papovaviruses, have also been shown to induce CNS tumors, but few models have been generated (Mukai & Kobayashi, 1973).

Cell culture or subcutaneous passage has maintained the tumors induced by either carcinogen or virus. By stereotactic localization, these cells or tissue can be injected with accuracy into a precise region of the brain. Transplantation can be made into a syngeneic host, but as the brain shows partial immune privilege, many models were generated by heterotransplantation into a non-syngeneic animal.

The C6 glioma was induced in randomly bred Wistar rat progeny by the transplacental effects of NNU. This tumor was cloned and is now available as a continuous cell line (ATCC No. CCL107). This cell line exhibits astrocytic morphology and the glial cell markers, glial fibrillary acidic protein (GFAP) and S100 protein. It has been used extensively in vitro and in vivo where the tumor has shown invasion into surrounding parenchymal tissue, neovascularity, and necrosis. The major weakness of the C6 cell line is its immunogenicity. Because of its generation in an outbred rat, it has no syngeneic host and, therefore, is highly immunogenic (Trojan et al., 1993). Most recently, spontaneous regression without therapeutic intervention using this model has been demonstrated. This calls into question the use of the C6 glioma model to evaluate therapies in support of their use in a clinical trial (Beutler et al., 1999).

The extensively used 9L gliosarcoma was induced in inbred CD Fischer rats using repeat injections of NNMU.

The tumor was cloned and initially exhibited glial characteristics, but with serial passage, these characteristics changed to give this 9L gliosarcoma cell line the features of a sarcoma, which when transplanted lost the ability to invade the parenchyma. Its inability to invade the parenchyma made it ideal to study the effects of genes that modulate tumor angiogenesis. 9L gliosarcoma tumors expressing retrovirally transfected oncogenic factors such as *ras* were shown to be vascularized and capable of parenchymal invasion (Schwartz et al., 1991). Like the C6 cell line, there is no syngeneic rat strain for the 9L gliosarcoma. Therefore, it is highly immunogenic and unsuitable for survival studies to assess long-term therapeutic effects.

The transplacental exposure of ENU to CD Fischer fetal rats resulted in the generation of the RG2/D74 glioma. Two identical clones were cultured from this tumor in two different laboratories. Hence, the clone was designated two names, RG2 and D74. This cell line produces a tumor in syngeneic Fischer rats, which is invasive, giving uniform lethality, and has proved impossible to immunize against. This lack of immunogenicity makes it an attractive choice to model the effects of anticancer drugs, tumor suppressor, and immune activation genes (Tjuvajev et al., 1995). Using the same methodology, the F98 glioma was also induced in CD Fischer rats. The F98 cell line derived from this tumor is only weakly immunogenic in the syngeneic host, if at all. Based on its in vivo morphology, it has been designated as an anaplastic glioma with characteristics that closely resemble human glioblastoma, and like RG2, is a good model for evaluating brain tumor therapies.

Another syngeneic rat model was produced by repeated injection of NNMU into an inbred Lewis rat, giving rise to the CNS-1 glioma (Kruse et al., 1994). The CNS-1 cell line expresses both GFAP and S-100, and in vivo inoculation gives rise to an invasive tumor. More studies are required to test the immunogenicity of this relatively new model. However, survival times within the syngeneic Lewis rat have been shown in our laboratory of up to 1 month with a tumor load of 5–10,000 cells implanted into the striatum (Dewey et al., 1999). Recently, our laboratory has shown the CNS-1 model to be a useful model for evaluating the efficacy of gene therapy treatment using the HSV1-TK/GCV strategy (Dewey et al., 1999). Long-term survivors after HSV1-TK/GCV treatment showed widely distributed transgene expression of HSV1-TK throughout the brain parenchyma and chronic inflammation at the site of tumor implantation and viral delivery (Dewey et al., 1999; Cowsill et al., 2000).

5.2. Murine glioma models

5.2.1. VM Dk murine astrocytoma

The inbred mouse strain VM Dk is unusual in its propensity to spontaneously develop astrocytomas. From one of these astrocytic tumors, two cell lines, SMA-497 and SMA-560, have been derived and thoroughly characterized (Serano et al., 1980). Both cell lines are highly tumorigenic and will form tumors intracranially and subcutaneously in the syngeneic VM/Dk (H-2^b) strain. The cell lines are considered to be glial derived and express the glial markers GFAP, S-100, and glutamine synthetase. In addition, SMA-560 secretes immunosuppressive TGF-β characteristic of many human gliomas (Sampson et al., 1997). The resulting tumor is locally invasive and necrotising, making this animal model extremely useful for gene therapy studies.

5.2.2. GL261 murine glioma

The GL261 murine glioma is one of a number of murine tumors that were induced by the intracerebral implantation of methylcholanthrene (MCA) pellets. This tumor has been established as a cell line and can be grown subcutaneously and intracerebrally in vivo. The histology of the tumor is similar to a rare ependymoblastoma seen in children, with poorly differentiated, uniform, small, cuboidal cells with little cytoplasm. However, they demonstrate no signs of ependymal differentiation and their exact origin is uncertain. These cells are most often implanted into C57BL/B6 mice (H-2b). Their uncertain lineage has led to these cells being immunogenic, but even though they lack histological similarity with more common malignant human tumors, this has not stopped their wide usage to test therapeutic responses. However, favorable therapeutic responses with this model should be qualified using another spontaneous tumor that may more accurately mimic the intrinsic qualities of human tumors.

5.3. Heterologous xenograft brain tumor models

Heterotransplantation of the cells or tissue can also be achieved into an immune incompetent animal to produce a xenograft model. Subcutaneous human glioblastoma tissue has been successfully grown in this artificial environment, and is a widely used animal model. However, analysis has shown tumor heterogeneity possibly due to differing selection of certain cell subpopulations (Jones et al., 1981), and the inherent limitations of using gene therapy strategies in immune suppressed animals must also be considered.

5.4. Transgenic glioma models

The generation of good animal models harboring similar features of malignant gliomas in tumors is of paramount importance for the development of gene therapies. The need for good models in preclinical testing is pressing, as it may determine the outcome of the treatment, it would diminish the exposure of patients to inefficient approaches and it would allow the testing of several treatment strategies to identify the most effective one. The most salient features that these animal models should possess are glial tumor cells, tumor infiltration of the brain parenchyma, neovascular formation, areas of necrosis surrounded by

palisading of tumor cells, and progression from low-grade to high-grade malignancy. Ideally, a model that encompasses both the genetic and the clinical features of the disease would be needed to address both mechanisms of disease progression and also for testing novel therapies. Nevertheless, models that reflect the clinical features, but lack the genetic basis of disease and vice versa, would be equally valuable, depending on the issues the experimenter wishes to address.

The ability to insert foreign DNA to introduce specific genes into the germ line of mammals has paved the way for the development of a new generation of glioma model. Serendipitously, it was found that if the large T-antigen gene of the Simian monkey virus (SV40), under the regulation of its own promoter and enhancer sequences, was placed in transgenic mice, choroid plexus tumors were reproducibly generated, as this T antigen is capable of transforming cells (Brinster et al., 1984). Moreover, if a tissue-specific transcriptional control element was included, cell transformation and, hence, tumor formation arose in a tissue-specific manner. Using the SV40 promoter and the moloney murine sarcoma virus enhancer to control large T-antigen expression, mice that developed midline brain neoplasms at 18 weeks, comparable to primitive human medulloblastoma, were produced. These tumors displayed photoreceptor-like features expressing S-100 and rhodopsin. This induction of primitive tumor growth has allowed neural-derived cell lines to be cloned, the generation of which had been impeded by the post-mitotic nature of mature, differentiated neurons. Some exciting glioma models are appearing that use glial-directed expression of oncogenes. Expression of the neu oncogene, originally isolated from a rat glioblastoma, under the control of the myelin basic protein (MBP) transcription regulatory element, produced high-grade, necrotic glioma, presenting oligodendrocytic antigens (Hayes et al., 1992). It recently has been demonstrated that loss of a single tumor suppressor gene or overexpression of an oncogene are not sufficient to induce high-grade malignant gliomas (Kamijo et al., 1999). Combination of genetic lesions, i.e., mice double heterozygous for p53 and NF1, develop low/intermediate-grade gliomas (Reilly et al., 2000).

The transforming gene of the Rous sarcoma virus (RSV), v-src, under the control of the glial fibrillary acidic protein (GFAP) gene regulatory elements that is expressed in astrocytes, was used to generate a transgenic model of astrocytoma. This model allows astrocytoma development to be studied as the tumors progressed from small foci expressing vascular endothelial growth factor (VEGF) to a tumor with the morphology and growth characteristics of glioblastoma (Weissenberger et al., 1997). Overexpression of activated EGFR in a background of INK4a deficiency, induced high-grade gliomas when expression was targeted to cells expressing nestin early in postnatal brains (Holland et al., 1998). Few tumors resulted when expression of EGFR was targeted to cells expressing GFAP (Holland et al., 1998), suggesting that neural stem cells are more prone to being transformed by overexpression of EGFR in a background of INK4a deficiency. When Ras and activated Akt were targeted to nestin-expressing cells, similar results are obtained when compared with targeting Ras and activated Akt to GFAP-expressing cells (Holland et al., 2000).

High-grade gliomas were also generated when PDGF was delivered into early postnatal brains using a retroviral vector system (Holland et al., 2000; Uhrbom et al., 2000). These murine models provide valid histopathological models of human malignant glioma and could provide invaluable tools for preclinical testing of new treatment modalities for this devastating disease.

6. Clinical trials

Malignant gliomas are attractive targets for local gene therapy because of the restricted localization to the CNS and the absence of distant metastases. The discovery of some of the molecular pathways, which underlie glioma pathophysiology, has suggested many potential gene therapies. Recombinant adenoviruses (rAds) and retrovirus producer cell lines are the most popular gene therapy vectors used to date, delivered directly into the brain either intra- or peritumorally with the aid of MRI guidance (Fig. 6). High-capacity adenoviral vectors capable of delivering many transgene copies and regulatable elements are also being developed, which will make these viruses very attractive vehicles for CNS gene transfer. Adenoviruses are not inactivated in vivo by the complement system; they transduce non-dividing cells and lack the potential to cause insertional mutagenesis. In addition, they have a high efficiency of transduction and allow the insertion of large transgenic sequences, i.e., up to 30 kbp when using gutless systems (Umana et al., 2001). Retroviral packing systems have a lower infection rate in comparison with rAds, and can only accommodate smaller transgene inserts. However, they are also attractive candidates, as they cannot infect non-dividing cells. The gene transfer efficiency of retroviruses and adenoviruses has been assessed in human malignant glioma in vivo. The results from this study indicate that adenoviruses are more efficient than retroviruses in achieving in vivo gene transfer (Puumalainen et al., 1998). HIV-derived lentiviral vectors have potential as viral vectors, but production and safety issues need to be addressed before they can enter clinical trials. Herpes simplex virus vectors are able to transfer large inserts; however, the properties of its genome are not fully understood.

Clinical trials for glioma treatment, so far, have typically involved the use of 'suicide genes' and investigate the therapeutic transfer of the herpes simplex virus thymidine kinase gene (HSV-tk), followed by the administration of the prodrug ganciclovir (GCV). Expression in tumor cells of HSV-tk will render these cells susceptible to GCV, a

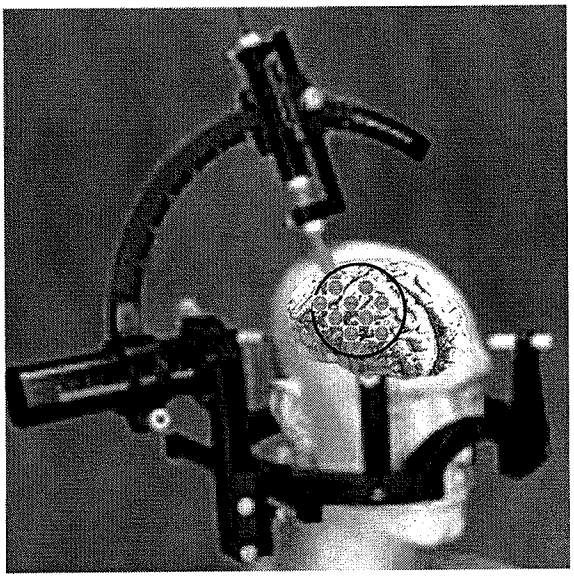

Fig. 6. MRI-guided stereotactic injection of adenovirus into CNS tumors in situ. Recombinant adenovirus vectors could be directly injected into multiple areas within the growing tumor or within the margins of a resected tumor using sterotactic injections and MRI guidance. Once injected, the viral vector will express the therapeutic transgene, i.e., HSV1-TK, within the tumor mass or surrounding tissue, thus allowing tumor cell destruction with the anti-herptic drug ganciclovir.

guanosine analogue (Moolten, 1986). Subsequent phosphorylation of GCV produces a toxic metabolite that blocks DNA replication (Matthews & Boehme, 1988), causing death of cells, which are proliferating, i.e., tumor cells. Incorporation of GCV-TP into DNA also results in chromosomal aberrations and sister chromatid exchange, subsequently leading to cell death (Thust et al., 1996). In addition, the cytotoxic effect of ganciclovir is further augmented when non-infected cells incorporate the phosphorylated bi-products of ganciclovir. This is known as "bystander effect" (Moolten, 1986).

To date, many of the human gene therapy clinical trials for the treatment of gliomas have involved the strategy of surgical resection of the tumor coupled with the injection of the vector either from an adenovirus or a retroviral vector, producing cells into the rim of the resected cavity (Klatzmann et al., 1998; Shand et al., 1999; Packer et al., 2000) (Fig. 7). Retroviral vector-producing cells expressing HSV-tk are in phase I/II trials for the treatment of glioblastoma in which the viral vector is delivered by injection to the resected tumor rim following surgery (Shand et al., 1999). The survival of patients with recurrent glioma treated in this way showed a similar median survival, 28–37 weeks (Klatzmann et al., 1998; Shand et al., 1999), compared with those studies involving resection and chemotherapy and/or other treatments. Trials involving direct injection of the virus into the tumor are more limited. In one trial, direct injection of the adenovirus encoding HSV-tk was performed and 3 of 13 patients survived more than 25 months (Trask et al., 2000).

Direct comparisons between adenoviral and retroviral gene therapy systems involving ganciclovir medication in malignant glioma are limited. Sandmair et al. (2000) reported a phase I trial involving three groups of seven patients in each group, all of which had primary or recurrent

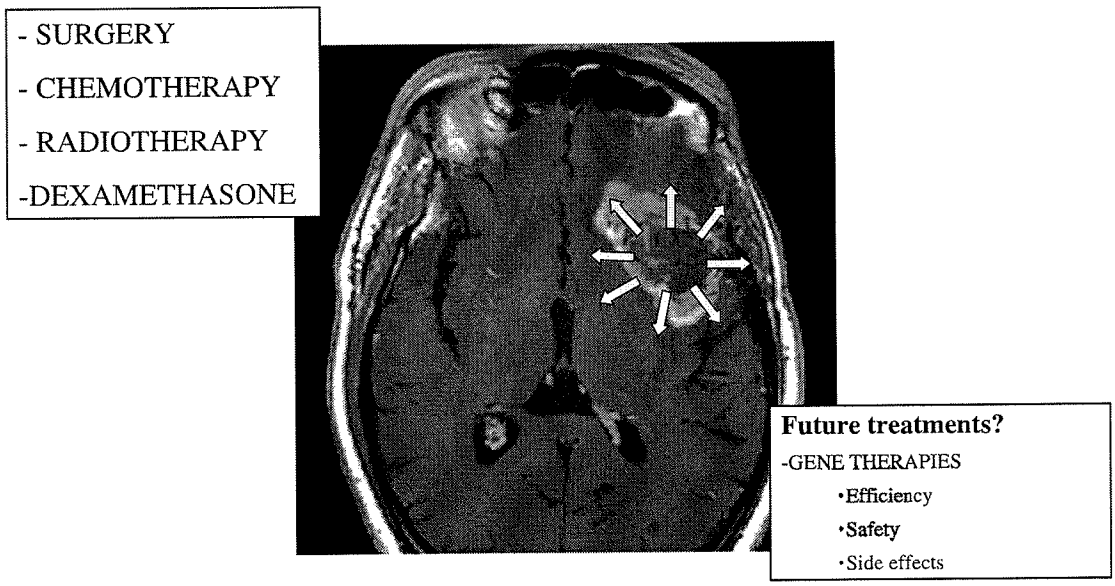

Fig. 7. Gene therapy delivery for the treatment of brain tumors. Gene therapy would be delivered in conjunction with classical treatment strategies, which include surgery, chemotherapy, and/or radiotherapy. Patients will also be treated with anti-inflammatory drugs to combat brain edema. The gene therapy vectors would be delivered either within the tumor mass or its margins after resection (yellow arrows). This would be done at the time of surgery. Gene therapies will need to be very closely monitored, both with respect to their efficiency, but also very importantly with respect to their safety and any putative adverse side effects.

Table 4
Clinical trials

Therapeutic gene	Delivery vehicle	Reference
HSV-TK	Adenovirus and Retroviral producer cells	Sandmair et al., 2000
HSV-TK	Adenovirus	Trask et al., 2000
HSV-TK	Retroviral producer cells	Trask et al., 2000
(n/a) replication, competent, viral vector	Herpes simplex virus, (HSV1716)	Kim, 2000
HSV-TK/IL-2	Retroviral producer cells	Palù et al., 1999
HSV-TK	Retroviral producer cells	Klatzmann et al., 1998
HSV-TK	Retroviral producer cells	Ram et al., 1997
HSV-TK	Adenovirus	Eck et al., 1996
HSV-TK	Retroviral producer cells	Kun et al., 1995
HSV-TK	Retroviral producer cells	Raffel et al., 1994
HSV-TK	Retroviral producer cells	Oldfield et al., 1993

lobar malignant glioma that could be removed surgically. The adenoviruses or retrovirus packaging cells were then injected under an operating microscope into the margins of the tumor cavity. Seven patients received retroviral packaging cells (PA317/tk), seven adenoviruses, and seven were treated with a control adenovirus encoding the marker gene β-galactosidase. Upon MRI evaluation, all of the tumors treated with retroviral packing cells had progressed after 3 months and showed similar survival times to the control group, 7.4 and 8.3 months, respectively. The adenovirus-treated group, in comparison, had a mean survival time of 15 months, and this difference was statistically significant, $P<0.012$ (Sandmair et al., 2000). The conclusion of the authors was that the results were encouraging, especially with respect to the adenoviral treatment, and they proposed that this treatment should from the basis of a randomized phase II trial.

From 1996 to 1998, the GL1328 international study group performed the first phase III study with retroviral packing cells expressing HSV-tk. Two hundred and forty-eight patients with previously untreated GB were entered into this trial. The patients were divided into two groups, and received either standard therapy or standard therapy with adjuvant gene therapy. No differences were detected in the recurrence free time, the onset of the progressive disease, and the overall survival of patients. However, no adverse effects relating to the injection of the retroviral packaging cells could be found (Rainov, 2000).

A replication competent mutant of herpes simplex virus, HSV1716, has also been used to treat glioma patients (Rampling et al., 2000). Direct stereotactic injections of the virus into the tumor have led to prolonged survival times, and 4 of 9 patients were disease free 14–24 months after the administration of HSV1716 (Rampling et al., 2000). Table 4 summarizes previous and ongoing clinical trials for human brain tumors.

As far as gliomas are concerned, long-term expression of therapeutic transgenes and greater stability of the viral vectors should lead to a more successful treatment of this tumor, where the rate of recurrence is very high and current treatment strategies are very ineffective. Also, with the development of vector systems that could be specifically targeted to the tumor and that can accommodate the use of several therapeutic transgenes and also regulatable promoter elements, it should be possible to provide novel gene-based therapies for this devastating neurological disease.

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